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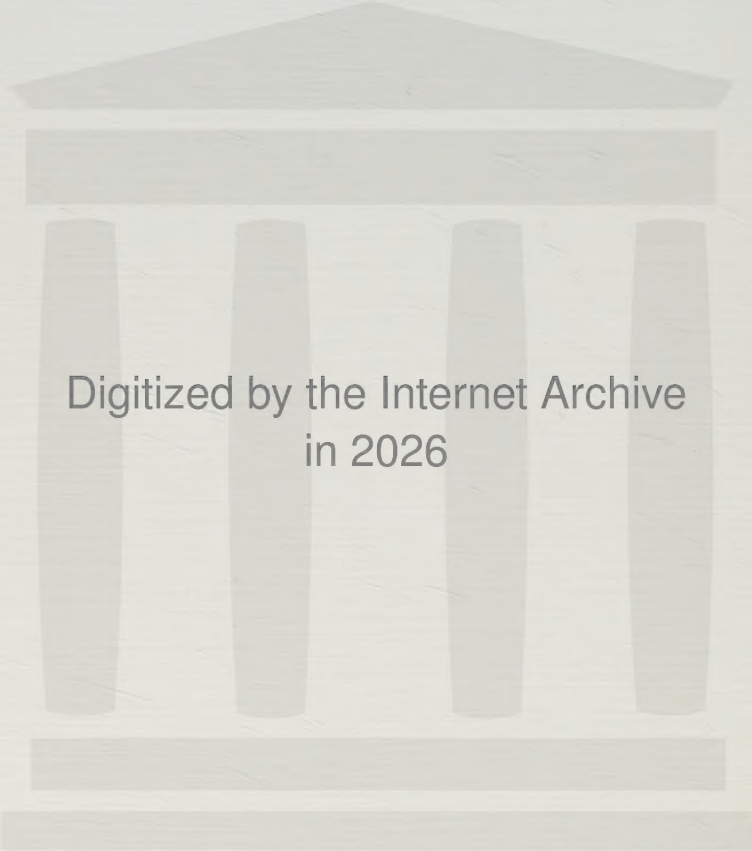
Cartilage Macromolecules

Isolation and characterization of
novel matrix constituents

Mats Paulsson



1983



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CARTILAGE MACROMOLECULES

Isolation and characterization of novel matrix constituents

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Abstract The cartilage extracellular matrix is composed of collagen, proteoglycans and non-collagenous proteins and is stabilized by interactions between, as well as within, these major groups of molecules. The non-collagenous proteins and a low-molecular-weight proteoglycan were studied, to gather some initial information about these less well known matrix components. A 148 kDa, non-collagenous protein was found to be a prominent constituent of bovine tracheal cartilage. It consists of three closely similar subunits connected by disulphide bonds and contains 4% carbohydrate. Cofractionation indicated that the protein is associated with proteoglycans. The 148 kDa protein is synthesized by the chondrocytes and was detected in varying amounts in most cartilages, with the notable exception of articular cartilage and the intervertebral disc. It was not detected in any non-cartilaginous tissues. With increasing age of the animal, a large proportion of the 148 kDa protein becomes insoluble to extraction with guanidinium chloride. A low-molecular-weight proteoglycan was identified in bovine cartilages and was purified using centrifugation in direct dissociative caesium chloride density gradients of low starting density. The monodisperse core protein has a characteristically high content of leucine and is substituted with one or two chondroitin sulphate chains, larger than those in other cartilage proteoglycans. The average molecular weight was 76000, when determined by sedimentation equilibrium centrifugation.			
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Studentlitteratur

Lund 1983

When Scaliger, whole years of labour past,
Beheld his Lexicon complete at last,
And weary of his task, with wond'ring eyes,
Saw from words pil'd on words a fabric rise,
He cursed the industry, inertly strong,
In creeping toil that could persist so long,
And if, enrag'd he cried, heav'n meant to shed
It keenest vengeance on the guilty head,
The drudgery of words the damn'd would know,
Doom'd to write lexicons in endless woe.

Samuel Johnson

(From the poem 'Know Yourself', written after
revising his famous lexicon of the English
language.)

CONTENTS

LIST OF PAPERS	5
ABBREVIATIONS	6
INTRODUCTION - FUNCTIONS AND MORPHOLOGY OF CARTILAGE	7
BACKGROUND - MACROMOLECULAR CONSTITUENTS OF THE	
CARTILAGE EXTRACELLULAR MATRIX	10
Proteoglycans	10
Collagen	15
Non-collagenous protein	17
Synthesis and turnover of cartilage matrix	
macromolecules	19
PRESENT INVESTIGATION - ISOLATION AND CHARACTERIZATION	
OF NOVEL CARTILAGE MATRIX CONSTITUENTS	21
Identification and some properties of major	
non-collagenous matrix proteins in tracheal	
cartilage	22
Purification and structural characterization of the	
148 kDa protein	24
Tissue distribution of the 148 kDa protein and its	
quantitative variation with age	25
Synthesis and turnover of cartilage proteins	28
Purification and structural characterization of a	
low-molecular-weight cartilage proteoglycan	30
CONCLUDING REMARKS	33
ACKNOWLEDGEMENTS	36
REFERENCES	38
APPENDIX: PAPERS I-VI	43

LIST OF PAPERS

This thesis is based on the following papers which are referred to by their Roman numerals.

- I. Matrix proteins bound to associatively prepared proteoglycans from bovine cartilage.
Mats Paulsson & Dick Heinegård
Biochem. J. 183, 539-545, (1979).
- II. Purification and structural characterization of a cartilage matrix protein.
Mats Paulsson & Dick Heinegård
Biochem. J. 197, 367-375, (1981).
- III. Radioimmunoassay of the 148-kilodalton cartilage protein.
Distribution of the protein among bovine tissues.
Mats Paulsson & Dick Heinegård
Biochem. J. 207, 207-213, (1982).
- IV. Variation in quantity and extractability of the 148-kilodalton cartilage protein with age.
Mats Paulsson, Sven Inerot & Dick Heinegård
In manuscript.
- V. Metabolism of cartilage proteins in cultured tissue sections.
Mats Paulsson, Yngve Sommarin & Dick Heinegård
Biochem. J. 212, 659-667, (1983).

VI. A novel, low-molecular-weight chondroitin sulphate proteoglycan isolated from cartilage.

Dick Heinegård, Mats Paulsson, Sven Inerot & Christian Carlström

Biochem. J. 197, 355-366, (1981).

ABBREVIATIONS

Gly - glycine

kDa - kilodalton

m.w. - molecular weight

res. - residues

S - sedimentation coefficient, expressed in Svedberg units

SDS - sodium dodecyl sulphate

INTRODUCTION - FUNCTIONS AND MORPHOLOGY OF CARTILAGE

Cartilage is like other connective tissues characterized by an abundant extracellular matrix and a relative sparsity of cells, i.e. chondrocytes. Almost all of the macromolecules in this matrix are synthesized by the chondrocytes and only a very minor portion is contributed by plasma proteins. The matrix is stabilized by a multitude of interactions between, as well as within, the major groups of molecules, i.e. collagen, proteoglycans and non-collagenous proteins. The important mechanical properties of cartilage, elasticity and resistance to compression, result from the physical characteristics of the individual matrix molecules and their interactions.

In early life most of the skeleton is cartilage. Thus one of the important roles for cartilage is to provide a scaffold for skeletal development, from which most of the non-cranial bones are formed through the process of endochondral ossification. Even though major portions of these cartilaginous precursors are rapidly replaced by bone tissue, cartilage remains at the epiphyses of the long bones. Here a growth plate is formed which allows longitudinal growth by the continued proliferation of chondrocytes. Each chondrocyte gradually becomes hypertrophic and its surrounding matrix is calcified. Growth ceases when the growth plate is completely replaced by bone, an event called closure of the epiphyses.

In the adult, cartilage is retained in a number of locations. In the joints and in the vertebral column it provides a cushioning effect, due to its elastic properties. It forms the articulating surfaces in joints, which by the properties of cartilage appear to be self lubricating (Armstrong & Mow, 1980). Cartilage also constitutes those parts of the supportive framework of the body, that because of the requirement for flexibility and elastic

deformability may not be calcified. Examples are the nasal septum, the tracheal rings, the outer ear, the larynx, the ventral part of the ribs and the intervertebral discs.

The morphology of cartilage varies somewhat with location and specialized function. Commonly a distinction, based on morphology, is made between hyaline, elastic and fibrous cartilages. Hyaline cartilage, named after its semitransparent gross appearance, is the major group, consisting of e.g. articular, nasal, tracheal and laryngeal cartilages. Elastic cartilage, forming the outer ear and the epiglottis, is the only cartilage that contains elastic fibers. Fibrous cartilage, as in the menisci and the annulus fibrosus of the intervertebral discs, is truly fibrous in gross appearance and accordingly contains an abundance of collagen. All types of cartilage have some features in common. For example, they have no innervation and contain no or few blood vessels and, with the exception of articular cartilage, they are all surrounded by a perichondrium, a layer of specialized fibrous connective tissue.

The chondrocytes, scattered throughout the cartilage, are enclosed by a narrow rim that shows different staining properties from the rest of the matrix. This rim forms the border of the lacuna, which in the intact tissue is completely filled by the cell and its pericellular matrix (Stockwell, 1979). Each lacuna may contain one or a few cells that form a clone, i.e. they are all derived from the same progenitor chondrocyte (Szirmai, 1969). When certain histological stains are employed, an intensely basophilic zone can be seen around the lacunae. This is referred to as the territorial matrix and is according to some investigators surrounded by a 'basket' consisting of a dense collagen meshwork (Szirmai, 1969), schematically outlined in Figure 1. Outside, the less basophilic interterritorial matrix makes up the major part of the tissue in most cartilages.

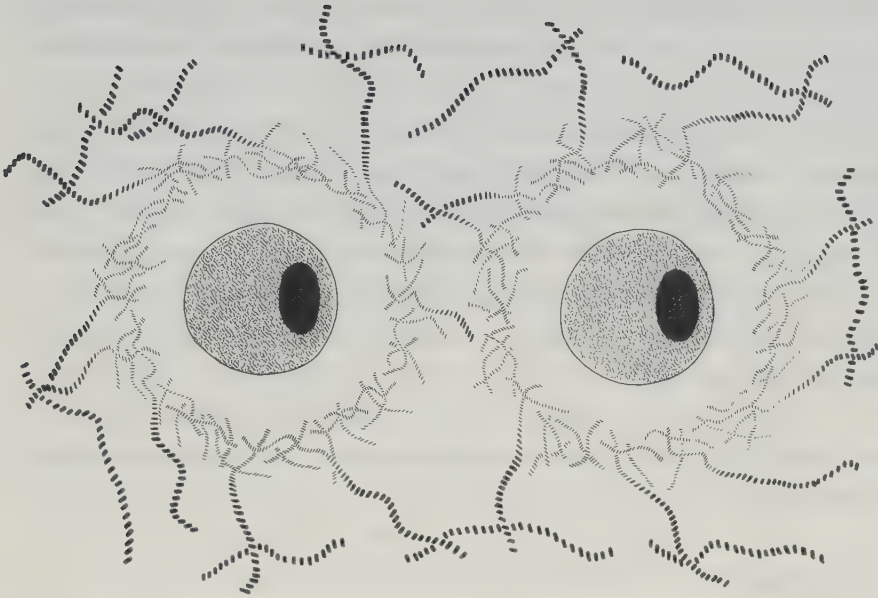


Figure 1. Schematic representation of the chondron concept, showing the chondrocytes in the territorial matrix, which is demarked from the interterritorial matrix by a 'basket' of denser collagen meshwork.

Early investigators suggested that the chondrocyte or chondrocytes present in one lacuna, together with the surrounding territorial matrix, may form a functional subunit of cartilage, a chondron, enclosed by the 'basket' structure (Benninghoff, 1922, 1925). Each chondron was thought to represent a separate tissue module, both from a mechanical and a structural standpoint. The concept is interesting in that it implicates specialized regions within the matrix. The existence of a 'basket' structure has, however, been challenged in more recent studies (Clarke, 1974).

As a consequence of the organization of cartilage, nutrition of the cells presents a special problem. All nutrients, hormones etc. have to reach the chondrocytes by diffusion through the matrix. In cartilages that are frequently compressed, e.g. articular cartilage and intervertebral disc, this process might be aided by regular flow of tissue fluid, which leads to transport of nutrients (McKibbin and Maroudas, 1979). Anyhow, the permeability properties of the extracellular matrix are important for the nutrition of the chondrocytes. This is one way in which the structure of the extracellular matrix may affect and control chondrocyte function.

BACKGROUND - MACROMOLECULAR CONSTITUENTS OF THE CARTILAGE EXTRA-CELLULAR MATRIX

Proteoglycans

Proteoglycans consist of a central protein core to which a variable number of glycosaminoglycan chains are attached. Many proteoglycans are in addition substituted with oligosaccharides. As the glycosaminoglycans carry large numbers of sulphate and carboxyl groups, the proteoglycan has a high negative net charge. Electrostatic repulsion between the glycosaminoglycan chains will cause the proteoglycan to assume an extended shape. The molecules therefore occupy very large domains, at least when in dilute solution (Hascall and Sajdera, 1970; Pasternack et al., 1974; Reihanian et al., 1979).

Proteoglycans contribute about a third of the dry mass of a hyaline cartilage. At the high concentration in the tissue they are much compressed. Presumably they are kept in this compressed state by physical entrapment in the meshwork of collagen fibers. The electrostatic repulsion between the fixed charged groups of the proteoglycans will be strong and any further decrease in

their domains will be resisted (Hascall and Hascall, 1981). By this effect, the proteoglycans contribute compressive stiffness and elasticity to the cartilage.

Early studies of proteoglycan structure were hampered by difficulties in obtaining representative samples of intact molecules with the low-salt-solvents that were used. A methodological breakthrough occurred through the work of Hascall and Sajdera, who devised an extraction procedure using high concentrations of chaotropic salts such as guanidinium chloride or magnesium chloride (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969). These solvents gave both a high extraction yield (80-90% of tissue proteoglycans) and protection of proteoglycans against degradation by tissue proteinases. The proteoglycans were purified by equilibrium centrifugation in caesium chloride density gradients, a method first applied to proteoglycans by Franek and Dunstone (1967). Somewhat later, porous agarose gel chromatography matrices became available, providing a useful tool for proteoglycan characterization (Heinegård, 1972; Hardingham & Muir, 1972a).

Using the new procedure for preparation it was found that cartilage proteoglycans form much larger particles when transferred from a dissociative environment, such as 4M guanidinium chloride, to an associative one, 0.4M guanidinium chloride (Hascall & Sajdera, 1969). The larger particles, proteoglycan aggregates, each contain about 50 proteoglycan monomers bound to a single molecule of hyaluronic acid, an interaction discovered by Hardingham and Muir in 1972(a). The interaction is mediated by a specific region of the proteoglycan core protein, the hyaluronic acid binding region (Heinegård & Hascall, 1974a), and has a K_D of 10^{-8} - 10^{-7} M (Christner et al., 1978; Cleland, 1979; Nieduszynski et al., 1980). Despite the already very strong bond, the interaction is further stabilized (Hascall & Heinegård, 1974;

Hardingham, 1979) by a third kind of molecule, the link protein, which binds to both the proteoglycan core protein and to the hyaluronic acid (Franzén et al., 1981; Tengblad, 1981). The link proteins are present in purified aggregates in a one to one ratio to proteoglycan monomers (Heinegård & Hascall, 1974a; Kimura et al, 1980). Three slightly different forms of this protein can be distinguished by SDS/polyacrylamide gel electrophoresis, with apparent molecular weights of 40-50 000 (Baker & Caterson, 1979a). The proteoglycan aggregate appears to be the dominating form of proteoglycan organization in cartilage.

Most studies of cartilage proteoglycan monomer structure have been performed on the large type of proteoglycan present in proteoglycan aggregates. These have molecular weights in the order of 2.5×10^6 (Hascall & Sajdera, 1970), but show a marked polydispersity as well as heterogeneity (see below). The average molecule contains about 100 chondroitin sulphate chains and 15-30 keratan sulphate chains bound to the protein core by their reducing terminal. The protein core constitutes about 8% of the mass of the proteoglycan, thus having a molecular weight of about 200 000 (Hascall & Riolo, 1972).

Three distinct structural regions have been identified within the proteoglycan protein core. Situated at one end, the hyaluronic acid binding region (Heinegård & Hascall, 1974a) is not substituted with glycosaminoglycans, but does contain N-linked oligosaccharides, Figure 2 (DeLuca et al., 1980; Lohmander et al., 1980). It has a globular structure (Perkins et al., 1981), as opposed to the other parts of the protein core that are highly extended. By different methods molecular weight estimates ranging between 60 000 and 100 000 (Heinegård & Hascall, 1974a; Perkins et al., 1981; Tengblad, 1981) have been obtained for this region. The chondroitin sulphate rich region is found at the other end of the protein core. It contains most of the chondroitin sulphate

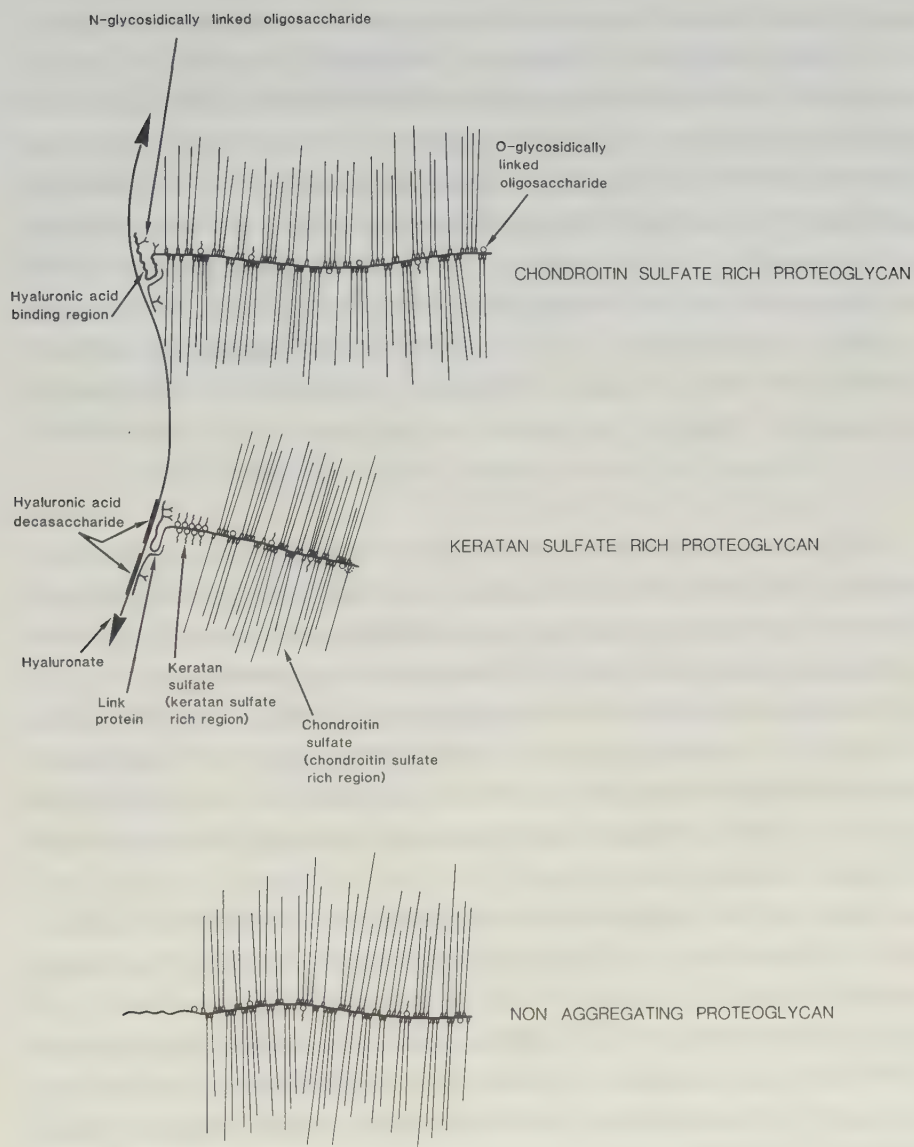


Figure 2. Structure of the three subpopulations of large cartilage proteoglycans.

chains and some of the keratan sulphate (Heinegård & Axelsson, 1977). The chondroitin sulphate is arranged in clusters of up to ten chains (Heinegård & Hascall, 1974b). Between the clusters are segments of unsubstituted core protein. The chondroitin sulphate rich region is of variable length (Heinegård, 1977), probably because of proteolytic trimming occurring while the molecules are in the matrix. In an average preparation it contains about half of the protein in the proteoglycan. The third region, the keratan sulphate rich region, is placed between the other two and contains most of the keratan sulphate chains in a very dense arrangement, Figure 2 (Heinegård and Axelsson, 1977). The protein part of this region has a molecular weight of about 20 000.

For some time there have been indications that cartilage proteoglycans are not only polydisperse but also heterogenous. The most striking evidence is perhaps the observation of two to three very sharp bands when cartilage proteoglycans are resolved by composite agarose/polyacrylamide gel electrophoresis (McDevitt & Muir, 1971; Stanescu et al., 1977). The concept of subpopulations was pursued by Heinegård and coworkers who isolated three distinct, large proteoglycans from nasal cartilage, Figure 2. Two of these contain the hyaluronic acid binding region and have the capacity to form aggregates (Heinegård et al., 1981a; D. Heinegård, J. Wieslander & J. Sheehan, unpublished results). They are, however, distinguished by their relative contents of the two glycosaminoglycans. One, the chondroitin sulphate rich proteoglycan, has a predominant chondroitin sulphate rich region and contains little keratan sulphate. The keratan sulphate rich region is poorly defined. The other, the keratan sulphate rich proteoglycan, does on the contrary contain keratan sulphate in a well defined keratan sulphate rich region. The core proteins of these proteoglycans show only partial immunological identity, which indicates that they differ not only in glycosylation.

The third subpopulation, the non-aggregating proteoglycan, Figure 2, lacks affinity for hyaluronic acid (Heinegård & Hascall, 1979). No hyaluronic acid binding region is present, even though this population has a higher relative protein content than the other two subpopulations. The high protein content makes it unlikely that this proteoglycan should be a degradation product of any of the other two.

A fourth subpopulation consisting of low-molecular-weight proteoglycans (Heinegård et al., 1981b) will be discussed under Present investigation.

Collagen

Collagen, the most abundant protein in the body, contributes the major part of the fibrous element in cartilage. Collagens extracted from various tissues have distinct structural differences and should be considered as a family of genetically related molecules rather than as one entity. In recent years a number of previously unrecognized collagen types have been reported, in addition to the well known interstitial collagens. For a summary of presently known collagen types see von der Mark (1981).

The characteristic subunit of collagen fibers, tropocollagen, consists of three polypeptide chains, α -chains, joined together in a triple helix (Ramachandran and Kharta, 1954). Each α -chain has a molecular weight of about 95 000. The tropocollagen units form higher structures by side to side alignment in a quarter stagger fashion (Gross, 1956). Formation of collagen fibrils and their stabilization by intra- and intermolecular crosslinks requires that posttranslational modifications of the collagen α -chains have occurred, i.e. hydroxylation of proline and lysine. Hydroxyproline is essential for helix stability (Berg & Prockop, 1973), while hydroxylysine participates in the formation of crosslinks. In the triple helical region there is a typical

Gly-X-Y repeat sequence, where the Y position is often occupied by proline or hydroxyproline. The high content of glycine allows a tight packing of the α -chains, essential for the unique helix arrangement.

The predominant cartilage collagen, designated type II, was first described by Miller and Matukas in 1969. It consists of three identical $\alpha 1(\text{II})$ chains and has a high content of hydroxylysine when compared with most other collagens. This high level of hydroxylysine is also reflected in a comparatively extensive glycosylation, as the galactosyl and glucosylgalactosyl saccharides typical for collagen are attached to the hydroxylysine residues (Butler & Cunningham, 1966).

Cartilage collagen differs from collagen in, for example, bone and skin by forming relatively thin fibrils, ranging from 10 nm at birth to 60-80 nm in diameter in the adult (Silberberg et al., 1961). The fibrils have a random orientation in the matrix. This might be a functional adaptation, as the role of collagen fibrils in cartilage is to distribute the load and to entrap proteoglycan aggregates, rather than to withstand stretching, which is the case in many tissues that have a more uniform fibril orientation.

Recently there have been several reports of additional minor collagen types in cartilage (for refs. see von der Mark et al., 1982). It has become increasingly clear that the composition of cartilage collagen is much more complex than what was earlier believed, although it is not yet known how these additional collagens are organized, i.e. if they are part of the fibrous framework of the tissue or if they have a more specialized localization and function. The fact that they are present in comparatively small amounts could indicate that the latter is more likely.

Non-collagenous protein

Already in the 1880's Mörner recognized that in addition to collagen and 'chondroitin sulfuric acid' cartilage also contains a fraction of non-collagenous proteins, which he called the 'albumoid' (Mörner, 1888, 1889). Even though fragments of the proteoglycan core protein were probably present in this fraction, such fragments can not account for more than a portion of the 'albumoid'. While the study of collagen and proteoglycans was pursued and gradually became more refined, the 'albumoid' was mostly neglected, a fact that was noted by Szirmai who in 1969 wrote the following:

'It should be pointed out in this connection that the proteins of the proteoglycan complex represent only a relatively small proportion of the total non-collagenous protein in cartilage. In the nasal septum cartilage, for example, quantitative analysis revealed that the values for a not yet characterized protein can range from 15 to 50% of the total dry weight (Szirmai et al. 1967). Little is known about the chemical composition and microscopical localization of Mörner's 'albumoid'. Thinking of his hesitation to assign a definite location to the collagen and this 'albumoid' (cf. Mörner, 1888 and 1889), and considering the lack of any chemical information on the nature of a protein which might constitute as much as one half of the total organic mass of cartilage, one can not escape the impression that our progress concerning some aspects of the cartilage structure has been remarkably slow.'

Since then some progress has been made, even though the knowledge of cartilage non-collagenous proteins is still minimal, when compared with the information available on the proteoglycan and collagen components or on non-collagenous proteins in some other forms of connective tissue.

In 1972 Keiser et al. first applied SDS/polyacrylamide gel electrophoresis to various fractions obtained from cartilage extracts. They observed the presence of a limited number of proteins in crude fractions. Two of these proteins were also present in proteoglycan preparations obtained by associative caesium chloride density gradient centrifugation, namely the two major link proteins. Their participation in proteoglycan aggregation was further ascertained by the finding of a large fragment of these molecules associated with hyaluronic acid and proteoglycan hyaluronic acid binding region in proteolytic digests of purified proteoglycan aggregates and of whole cartilage (Heinegård & Hascall, 1974a).

The two major link proteins have been purified to homogeneity and submitted to structural analysis (Baker & Caterson, 1979b; Bonnet et al., 1978). They have molecular weights of 46 000 and 40 500, respectively, but are very similar with regard to amino acid and carbohydrate composition. They do also yield similar patterns of proteolytic cleavage products (Baker & Caterson, 1979b). A third component of somewhat lower molecular weight has been identified more recently (Baker & Caterson, 1979a). As this third link protein has the same mobility on SDS/polyacrylamide gel electrophoresis as the major fragment produced by limited trypsin digestion of the two larger link proteins, it is believed to be a physiological degradation product of these.

Chondronectin is another non-collagenous protein that appears to be present in cartilage. It was first identified in serum as a factor that mediates the attachment of chondrocytes to type II collagen substrates (Hewitt et al., 1980), in analogy with the fibronectin mediated attachment of fibroblasts to substrates. By the use of immunofluorescence microscopy, chondronectin has been shown to be present in the vicinity of the chondrocyte in cartilage sections and at the surface of cultured chondrocytes (Hewitt

et al., 1982). It is a glycoprotein consisting of subunits with an apparent molecular weight of 70 000. As the intact protein has been estimated to have a molecular weight of about 180 000, it is likely to consist of two or three such subunits.

Recently the isolation of a non-collagenous protein from guanidinium chloride extracts of bovine foetal epiphyseal cartilage was reported (Choi et al., 1983). This protein is a dimer of 35 000 m.w. subunits and contains about 4% carbohydrate. The amount of the protein was found to increase with time during foetal development. In the third trimester it is the most prominent non-collagenous protein. By use of specific antibodies it was shown to be distinct from the link proteins and to be present in several types of cartilage.

Synthesis and turnover of cartilage matrix macromolecules

During growth and maturation connective tissue is continuously being remodelled. A prerequisite for remodelling is the turnover of extracellular matrix macromolecules; i.e. removal from the matrix and catabolism of 'old', perhaps damaged, molecules, proceeding in concert with synthesis of 'new' molecules and their incorporation into the matrix. Cartilage matrix turnover is most rapid at young age, but at least proteoglycans are metabolized also in the adult. The mechanisms for this process are only partially known. The biosynthesis of proteoglycans and collagen has been rather extensively studied, while there is less information about their assembly in the matrix and about their degradation and ensuing elimination. Again, there is no information about the turnover of non-collagenous proteins, except for limited data on the link proteins.

The first step in proteoglycan biosynthesis is the formation of the protein core. The primary gene product has been identified after translation of chondrocyte mRNA in cell free systems and is

a polypeptide of m.w. approximately 340 000 (Upholt et al., 1979; Treadwell et al., 1980). A core protein of similar size can also be isolated by labelling of chondrocytes with radioactive amino acids followed by immunoprecipitation of cellular protein (Kimura et al., 1981). The synthesis of chondroitin sulphate chains is initiated by transfer of xylose to serine residues on the core protein (Robinson et al., 1966), probably occurring in the rough endoplasmic reticulum. The consecutive transfer of two galactose and one glucuronic acid residue probably occurs in the smooth endoplasmic reticulum, whereafter the chain is elongated by the alternating addition of N-acetylgalactosamine and glucuronic acid, followed by sulphation of N-acetylgalactosamine residues (for refs. see Rodén, 1980). Concomitantly with the chondroitin sulphate synthesis, also keratan sulphate and oligosaccharides are added to the core protein. Very shortly after sulphation, the proteoglycan is secreted to the extracellular environment, where complexes of proteoglycan and link protein combine with hyaluronic acid to form stable aggregates (Kimura et al., 1979, 1980; Björnsson & Heinegård, 1981).

The proteoglycans in the matrix are gradually being degraded, probably due to the action of proteolytic enzymes (McDevitt et al., 1981) and the fragments formed either leave the tissue by diffusion or are taken up by the chondrocytes and further degraded in the lysosomes. The mean life of a proteoglycan varies with the species and the age of the animal, but in adult cartilages it ranges from months to years (Maroudas, 1979).

Collagen is synthesized in a precursor form, procollagen. In addition to the major triple helical portion that will form the tissue collagen, procollagen contains N- and C-terminal propeptides (for refs. see Fessler & Fessler, 1978). After synthesis on the ribosome the α -chain of procollagen undergoes extensive posttranslational modifications. An example of this is the

formation of hydroxyproline and hydroxylysine, that occurs already in the rough endoplasmic reticulum. Some of the hydroxylysine residues become glycosylated with mono- or disaccharides and the C-terminal propeptide becomes substituted with larger oligosaccharides. Three pro α -chains are then linked together by disulphide bonds between the C-terminal propeptides. Eventually triple helix formation occurs, but probably not until in the Golgi complex (Olsen, 1981).

After secretion from the cell, both the N- and C-terminal propeptides are split off by specific procollagen proteases (for refs. see Bornstein & Traub, 1979). This allows the newly formed collagen to participate in fibril formation. The fibrils gradually become stabilized by intra- and intermolecular covalent crosslinks. Once formed, cartilage collagen fibers are extremely stable. As an example, in normal adult human articular cartilage there is virtually no turnover of collagen, i.e. the turnover time of the collagen has been calculated to be about 400 years (Maroudas, 1979). In some experimental animals, however, higher turnover rates have been measured (Repo & Mitchell, 1971), so the rate may vary with species and location as well as with age. The mechanism of elimination is proteolytic degradation and both specific collagenase and less specific proteases have been implicated.

PRESENT INVESTIGATION - ISOLATION AND CHARACTERIZATION OF NOVEL CARTILAGE MATRIX CONSTITUENTS

When the present study was initiated, an increasing knowledge of the higher organization of both cartilage proteoglycans and collagen had allowed the design of structural models. Despite this, our concepts of the overall organization of the cartilage matrix were still vague and included many unproven assumptions.

To construct an integrated model for the molecular organization of cartilage extracellular matrix, though, all of the participating molecular components have to be known. As pointed out by Szirmai (1969) in the quotation above, one group of molecular constituents of the matrix, the non-collagenous proteins, had been neglected. In addition, our knowledge about proteoglycans and collagen was largely limited to those molecules obtained by the use of the classical preparation procedures, outlined by Hascall & Sajdera (1969) for proteoglycans and by Miller (1972) for collagen. These methods give preparations that are representative for the bulk of the proteoglycans and collagen, but minor, although potentially important, populations of structurally different molecules may be lost in side fractions.

The present study was undertaken to fill some of the gaps in our knowledge of such matrix macromolecules that might be components of the structural framework in cartilage. The effort was primarily directed towards the non-collagenous proteins, but also included such proteoglycans that are not recovered when the conventional preparation procedure is used. At about the same time, a similar route was taken by workers in the collagen field, which has led to the identification of a new set of collagenous molecules in cartilage (for refs. see von der Mark et al., 1982).

Identification and some properties of major non-collagenous matrix proteins in tracheal cartilage (paper I)

The search for novel non-collagenous matrix proteins was initiated by chromatographic and electrophoretic analysis of whole extracts of bovine tracheal cartilage. One goal was to identify matrix proteins interacting with other matrix constituents, primarily proteoglycans and/or collagen. Extraction with chaotropic salts, such as guanidinium chloride, might denature proteins irreversibly, with the result that interactions occurring between native matrix molecules are not recognized. Such

solvents were therefore initially avoided. Instead high speed homogenization in a physiological buffer was used to solubilize a fraction of the proteoglycans and proteins from the tissue. The homogenization residue was then extracted with 4M guanidinium chloride.

SDS/polyacrylamide gel electrophoresis revealed a few unknown proteins, in addition to the link proteins. The most abundant polypeptide had an apparent molecular weight of 60 000, when electrophoresed after reduction. Without reduction it was present in a much larger complex and was seen close to the top of the gel. This indicated a disulphide-dependent oligomeric structure. The protein was present both in the homogenate and in the subsequent guanidinium chloride extract. It was later designated the 148 kDa cartilage protein.

Another protein gave a sharp band with an apparent molecular weight of about 36 000. It had the same mobility with and without prior reduction. Only small amounts were present in the homogenate, while it was enriched in the guanidinium chloride extract. This protein we designate the 36 kDa cartilage protein.

When homogenate was chromatographed on Sepharose CL2B in 0.15M NaCl the 148 kDa protein coeluted with the proteoglycans and the link proteins. In 4M guanidinium chloride, though, most of the 148 kDa protein eluted separate from and more retarded than the proteoglycans. When a sample was dialysed into 4M guanidinium chloride and then back to 0.15M NaCl, the 148 kDa protein again coeluted with the proteoglycans. Cofractionation between the 148 kDa protein and the proteoglycans was also seen in rate-zonal centrifugation and in ion-exchange chromatography. Since these methods separate according to different molecular characteristics, the 148 kDa protein was assumed to interact with proteoglycans.

Purification and structural characterization of the 148 kDa protein (paper II)

The 148 kDa protein was purified from guanidinium chloride extracts of bovine tracheal cartilage by the use of equilibrium centrifugation in caesium chloride density gradients followed by gel chromatography on Sephadex G200 and Sepharose CL6B, all in the presence of 4-5M guanidinium chloride. Final purification was achieved by dialysis into 0.1M guanidinium chloride, where the 148 kDa protein is not soluble and precipitates quantitatively. Subunits of the protein were prepared by reduction and alkylation.

The molecular weights of the intact protein and of the reduced and alkylated subunits were determined by sedimentation equilibrium centrifugation in 6M guanidinium chloride and were found to be 148 000 and 52 000, respectively, showing that the protein is composed of three subunits of similar size. Even though the trimeric structure could be taken to indicate a derivation from procollagen, the molecular weight of the molecule is too different from that of any of the physiological split products of procollagen for this to be plausible.

The molecular weights determined by analytical ultracentrifugation were in poor agreement with those estimated by SDS/polyacrylamide gel electrophoresis. Construction of a Ferguson plot (Ferguson, 1964) showed that the 148 kDa protein subunits had a higher apparent free electrophoretic mobility than reference proteins, possibly due to abnormally high binding of SDS. This invalidates the molecular weight assessments by SDS/polyacrylamide gel electrophoresis.

Chemical analysis showed that aspartic acid/asparagine and glutamic acid/glutamine are predominant amino acids in the 148 kDa protein. Hydroxyproline is absent, proving the protein non-collagenous. The carbohydrate content is 3.9% and glycopeptides, prepared by papain digestion followed by gel chromatography on Biogel P10, were rich in glucosamine and mannose. Furthermore, the glycopeptides were enriched in aspartic acid/asparagine compared with the intact protein, indicating that the oligosaccharides are mostly of the N-linked type, typical for glycoproteins.

Tissue distribution of the 148 kDa protein and its quantitative variation with age (papers III & IV)

The pure 148 kDa protein was used for the preparation of a specific antiserum. A radioimmunoassay was developed, which allowed quantitation of as little as 10 ng/ml of the protein. Difficulties caused by the low solubility of the 148 kDa protein in conventional buffers were overcome by the use of SDS in samples and in solutions of ^{125}I -antigen. Excess SDS was bound in mixed micelles with Triton X100 to avoid denaturation of antibodies. Even though the assay was performed in the presence of SDS, it appears that the folding of the protein is important for antibody recognition, since most of the antigenicity was lost after reduction and alkylation of the 148 kDa protein.

The assay was used to determine the presence of the 148 kDa protein in guanidinium chloride extracts of a large number of bovine tissues. The protein was detected only in extracts of cartilage. Among these there were marked differences in the amount of 148 kDa protein present. The largest quantities were found in tracheal cartilage, where this protein alone contributed at least 4% of the tissue dry weight. The protein was also present in extracts of nasal septum, xiphisternal, auricular and

epiphysial cartilage, but in lower quantities. Surprisingly, it could be detected neither in extracts of articular cartilage nor of the intervertebral disc.

The distribution of the 148 kDa protein was confirmed by SDS/polyacrylamide gel electrophoresis. This technique also gave some information on the distribution of other cartilage proteins among tissues. The link proteins and the 36 kDa protein were present in all cartilages. Extracts of articular cartilage and intervertebral disc, while lacking the 148 kDa protein, contained other predominant proteins that were not present in most cartilages. Some non-collagenous proteins, then, such as the link proteins and the 36 kDa protein are probably common to all cartilages, while others, like the 148 kDa protein, are subject to variation and might be markers of differentiation among cartilages.

The samples used to study the tissue distribution were taken from cattle of about two years of age. To determine if the contents of matrix proteins vary with the age of the individual, a set of tracheal cartilage samples were taken from steers ranging from fetuses to an animal 12 years of age. In guanidinium chloride extracts marked differences were detected, particularly with regard to the 148 kDa protein, which increased in quantity more than five-fold between birth and four years of age. At higher ages there was a gradual decrease in the amounts extracted.

Extractability of, for example, proteoglycans is known to decrease with increasing age (Inerot & Heinegård, 1983) and the decrease in 148 kDa protein at high ages could in analogy be due to low extraction yields. We therefore tried to determine if some of the 148 kDa protein is left in the extraction residue and if so, if this fraction increases at high age. This was possible

since some proteases, that solubilize at least part of the extraction residue, do not severely affect the antigenicity of the 148 kDa protein, even though the protein is partially degraded.

A large, additional fraction of the protein could indeed be released by trypsin digestion of the residue. Also in the trypsinate, there was an initial increase in the amount of 148 kDa protein, but the increase was somewhat delayed, when compared with the guanidinium chloride soluble pool, and the maximum was reached first at about eight years of age. At this age about six times more 148 kDa protein was detected in the trypsinate than in the guanidinium chloride extract. At even higher ages the amount of 148 kDa protein decreased also in the trypsinate.

An attractive interpretation is that the guanidinium chloride soluble pool of 148 kDa protein is a transient one, consisting of comparatively recently secreted molecules, that will eventually be laid down in the matrix in a manner that renders them insoluble in guanidinium chloride. The mechanism by which the 148 kDa protein becomes insoluble is not known, but it is possible that, in analogy with collagen, it may depend on the formation of covalent crosslinks.

For comparison, the amounts of link protein in the guanidinium chloride extracts were determined. This protein showed a slight, continuous increase in quantity with age, but far from the dramatic changes seen with the 148 kDa protein. Neither did the amount of 36 kDa protein change markedly with age. An increased insolubility with age does not appear to be a general phenomenon among non-collagenous matrix proteins.

The addition of a temporal aspect to the occurrence of the 148 kDa protein and the discovery of the guanidinium chloride insoluble pool raises some new questions with regard to the tissue distribution of the protein. It might be present also in articular cartilage, but then only in animals older than those used for the study of tissue distribution or it might escape detection due to insolubility in guanidinium chloride. Those aspects are presently being investigated.

Synthesis and turnover of cartilage proteins (paper V)

Most extracellular components of cartilage are synthesized by the chondrocytes, but the possibility of diffusion of macromolecules from surrounding tissues can not a priori be excluded. It was therefore important to determine the place of synthesis for the 148 kDa and 36 kDa proteins. Another goal for the study was to find out if the non-collagenous proteins, such as the 148 kDa and 36 kDa proteins, have a slow turnover like collagen and cartilage proteoglycans.

In initial experiments we tried to identify the 148 kDa and 36 kDa proteins in cultures of isolated bovine tracheal cartilage chondrocytes, grown either as primary cells in suspension or in monolayer. A similar system has earlier been used to study proteoglycan synthesis (Björnsson & Heinegård, 1981). We could, however, identify neither the 148 kDa nor the 36 kDa protein in the media or in guanidinium chloride extracts of the cells. The pattern of ^3H -leucine labelled proteins seen on SDS/polyacrylamide gel electrophoresis was quite different from the protein composition in extracts of whole tissue.

In search for a more 'physiological' culture system we then turned to organ culture of sections of bovine tracheal cartilage. The patterns of radiolabelled proteins in guanidinium chloride extracts of the cultured sections differed from those obtained

from isolated cells and were more similar to the composition seen in extracts of unlabelled cartilage. The 148 kDa protein, the 36 kDa protein and the link proteins were major labelled components in the section cultures.

More than 99% of the incorporated ^3H -leucine was retained in the cartilage, but only about 50% of the labelled molecules could be extracted with 4M guanidinium chloride. The nature of the unextractable material remains uncertain. There was a lag time of 20-25 minutes, after the start of a ^3H -leucine pulse, until the linear accumulation of guanidinium chloride extractable macromolecules began. As the equilibration of ^3H -leucine was rapid, this was taken to indicate that proteins do not become extractable until after secretion from the cells and therefore that the bulk of the molecules in the guanidinium chloride extracts were extracellular. In previous work it has been shown that ^{35}S -labelled proteoglycans also display a lag time before becoming extractable and that this corresponds to the time for their secretion from the cells (Hardingham & Muir, 1972b).

Proteins and proteoglycans in extracts of radiolabelled cultures were separated by caesium chloride density gradient centrifugation in 4M guanidinium chloride and the proteins were further fractionated by SDS/polyacrylamide gel electrophoresis. Only about 5% of the macromolecular ^3H -leucine in the extracts was recovered with the proteoglycans. Considerably more ^3H -leucine was incorporated into the 148 kDa and 36 kDa proteins than into the proteoglycan core protein.

The turnover of macromolecules was studied in experiments where a pulse of ^3H -leucine was followed by a prolonged chase in non-radioactive medium. The 148 kDa protein was found to have a very slow turnover, similar to that of the proteoglycans. The 36

kDa protein was eliminated more rapidly, i.e. radioactivity in this protein had decreased to about half during a chase of 188 hours.

It was concluded that both the 148 kDa and 36 kDa proteins are synthesized by the chondrocyte and are indeed major biosynthetic products of the tracheal cartilage. The 148 kDa protein displays the very slow turnover that is typical for structural matrix components. Also, it appears that synthesis of these proteins is suppressed in isolated chondrocytes and that an intact matrix is required for a 'normal' chondrocyte function.

Purification and structural characterization of a low-molecular-weight cartilage proteoglycan (paper VI)

In the established preparation scheme for cartilage proteoglycans (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969) non-aggregating proteoglycans having a comparatively low glycosaminoglycan substitution will be lost because of their low charge density to the upper part of the caesium chloride gradient. The top part of the gradient is heavily contaminated with protein and is therefore usually discarded. To be able to study also those proteoglycans, we wanted to design a procedure to isolate a larger proportion of the total extractable proteoglycans. To avoid preparation artifacts, it was important to prevent activity of degrading enzymes. Both these aims were achieved by direct centrifugation of the extract in 4M guanidinium chloride at the low starting density of 1.34 g/ml. The density obtained in the bottom fraction is then about the same as that in the top fraction of a dissociative gradient with the normal starting density of 1.52 g/ml.

The characterization of the proteoglycan preparation included SDS/polyacrylamide gel electrophoresis of the fractions from the gradient centrifugation. When samples were pretreated with

chondroitinase ABC, a band was seen in gels corresponding to fractions of intermediate density, where proteins would not be expected to be present. If the chondroitinase digestion was omitted, this band was absent, indicating that it must represent the core protein of a proteoglycan. To enter the 8% polyacrylamide gels it must be considerably smaller than the core proteins of the major cartilage proteoglycans. The core protein band was quite homogenous and migrated to approximately the same position as the link proteins, corresponding to an apparent molecular weight of 40-50 000.

By using the core protein as a marker, this novel proteoglycan could be purified further in a sequence of gel chromatographic steps. Even though the preparation was not completely homogenous, it was possible to estimate the molecular weight to about 70 000 by sedimentation equilibrium centrifugation.

Larger quantities of the proteoglycan in higher purity were prepared by an alternative route. The low density proteoglycans contained in the top fraction from conventional associative caesium chloride density gradients were recentrifuged in dissociative caesium chloride gradients of low starting density. Proteoglycans obtained in the bottom fraction were further fractionated by rate-zonal centrifugation in sucrose gradients and Sepharose CL4B chromatography in 4M guanidinium chloride. The final preparation was chromatographically homogenous and only the core protein band was seen on SDS/polyacrylamide gel electrophoresis after chondroitinase treatment.

The proteoglycan thus purified had a molecular weight of 76 000 by sedimentation equilibrium centrifugation in 4M guanidinium chloride. The apparent partial specific volume was assessed to be 0.59 ml/g and the $S_{20,w}^0$ value was 3.11 S. It did not show any affinity for hyaluronic acid.

The amino acid composition of the low-molecular-weight proteoglycan was drastically different from those of large cartilage proteoglycans, particularly in the very high content of leucine (143 res./1000 res.) and the comparatively low contents of serine and threonine. Protein contributed 25% of the total weight, while chondroitin sulphate chains and oligosaccharides made up the remainder. The chondroitin sulphate chains were of larger size than those in other cartilage proteoglycans, further substantiating that the low-molecular-weight proteoglycan is not a degradation fragment.

By combining the data on molecular weight and protein content, the likely molecular weight of the core protein should be around 20 000. The retardation coefficient obtained in a Ferguson plot (Ferguson, 1964) constructed from SDS/polyacrylamide gel electrophoresis data obtained at varying polyacrylamide concentrations, did, however, indicate a molecular weight of 42 000 for the core protein, prepared by chondroitinase digestion. Remaining chondroitin sulphate stubs and oligosaccharides can only partially account for the discrepancy. The apparent free electrophoretic mobility, though, was lower than should be expected from the retardation coefficient, when compared with reference proteins. This indicates non-ideal electrophoretic behaviour (Banker & Cotman, 1972) and renders the size estimate by gel electrophoresis unreliable. The observation of this anomaly is important, as SDS/polyacrylamide gel electrophoresis is often used to assess molecular weights of proteoglycan core proteins (and even of whole proteoglycans!) by comparison with globular reference proteins and without stringent analysis of the ideality of the electrophoretic behaviour.

The low-molecular-weight proteoglycan was identified also in bovine tracheal and articular cartilages and might thus be a general feature of cartilage. Proteoglycans of similar size have been observed as radiolabelled components in media from chondrocytes and in cartilage tissue culture (Kasarawa et al., 1979; Vasan & Lash, 1979). In parallel with our purification of the low-molecular-weight proteoglycan from bovine nasal cartilage, Stanescu and Sweet (1981) isolated a similar molecule from baboon articular cartilage.

CONCLUDING REMARKS

Much further work will be required to identify the exact biological function of the molecules described in the present text. Both the 148 kDa protein and the low-molecular-weight proteoglycan are likely to be structural components, but what are their exact roles in the overall organization of the cartilage matrix? Early steps in the elucidation of their functions must be the determination of their localization within the matrix and further study of their interactions with other matrix macromolecules.

With regard to the 148 kDa protein, preliminary data from immunofluorescence microscopy indicate that this protein is present throughout the extracellular matrix in bovine tracheal cartilage, with the possible exception of the pericellular zone, Figure 3 (B. Cederholm, M. Paulsson & D. Heinegård, unpublished results). The staining often has a 'patchy' character, possibly indicating local accumulations of the protein. Another, rather intriguing observation with possible bearing on the organization of the 148 kDa protein, was obtained by electron microscopy of samples of the purified protein. When renaturation is attempted, by slow, stepwise dialysis from guanidinium chloride solutions into physiological saline, a particulate suspension is formed.



Figure 3. Demonstration of the 148 kDa protein in the extracellular matrix of bovine tracheal cartilage by indirect immunofluorescence.

The particles appear as bundles of thin filaments, after contrasting by negative staining or rotatory shadowing (B. Engfeldt, M. Paulsson & D. Heinegård, unpublished results). Possibly, this in vitro phenomenon illustrates an inherent characteristic of the protein. As described in paper I, the 148 kDa protein persistently cofractionates with proteoglycans, when isolated in physiological buffers. So far our attempts to determine the nature of the interaction leading to this cofractionation have been fruitless. Perhaps selfassociation among the protein molecules, superimposed on an association with proteoglycans, is one of the causes for the inconclusive results.

Another issue that should eventually be addressed is the structural diversity among cartilage extracellular matrices. From the data presented in paper III it is clear that the non-collagenous protein is of dissimilar composition in different cartilages. If these proteins play a role in the structural organization of the matrix, the differences are probably reflected in other properties, such as the permeability and mechanical characteristics of the tissue. Perhaps the composition of the non-collagenous protein is an important variable in tailoring each type of cartilage extracellular matrix to the specific demands it encounters.

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47-59.

Matrix Proteins Bound to Associatively Prepared Proteoglycans from Bovine Cartilage

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Proteoglycans were extracted from bovine tracheal cartilage by high-speed homogenization, the use of dissociative solvents being avoided. The homogenate was fractionated by gel chromatography, sucrose-density-gradient centrifugation and ion-exchange chromatography. A previously unrecognized protein, cartilage matrix protein, was identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. It co-fractionated with the proteoglycans in all systems, indicating an interaction. The cartilage matrix protein–proteoglycan complex was dissociated by treatment with 4M-guanidinium chloride. The complex again formed when the guanidine was removed. The cartilage matrix protein has a mol.wt. of more than 200 000. On reduction it yields subunits with a mol.wt. of approx. 60 000.

During the last few years a more detailed picture of the molecular structure of cartilage proteoglycans and collagen has evolved. One of the main cartilage constituents, collagen, forms a fibrillar network in which aggregates of the other major constituent, cartilage proteoglycans, are entangled. The aggregates are formed by the specific interaction between hyaluronic acid and the protein-rich hyaluronic acid-binding region of the proteoglycan monomer (Hascall & Heinegård, 1974*a,b*; Heinegård & Hascall, 1974; Hardingham & Muir, 1972, 1973*a,b*). The interaction is stabilized by link proteins (Keiser *et al.*, 1972; Hascall & Heinegård, 1974*b*; Baker & Caterson, 1977; Caterson & Baker, 1978). Along one molecule of hyaluronic acid a large number of proteoglycan monomers can be bound.

In most studies of cartilage proteoglycans the dissociative preparation procedure designed by Hascall & Sajdera (1969) has been used. This involves extraction with concentrated solutions of chaotropic salts such as guanidinium chloride or $MgCl_2$, followed by separation of proteoglycans from other cartilage components by equilibrium centrifugation in a $CsCl$ gradient. Such procedures have been shown to give a proteoglycan preparation less degraded than the one obtained when proteoglycans were solubilized by high-speed homogenization in water or weak salt solutions, techniques commonly used earlier (Malawista & Schubert, 1958). The dissociative preparation procedure, however, has some inherent disadvantages, when detailed studies of the interactions forming the proteoglycan aggregate and the stoichiometry of the participating molecules

are attempted. Aggregates are obtained by recombining the dissociated components in the guanidinium chloride extract by lowering the guanidine concentration. It cannot be taken for granted that the recombined aggregates are in all features identical with the native ones. The spacing and the relative distribution of the constituents along the hyaluronic acid might be altered in the recombined aggregate. Proteins participating in the organization of the three-dimensional network of proteoglycans and collagen in the tissue might be irreversibly denatured or otherwise lost in the preparative procedure.

The purpose of the present study was to demonstrate cartilage proteins, other than the link proteins, capable of binding to proteoglycans. To avoid dissociative solvents proteoglycans were prepared by high-speed homogenization. The proteoglycans obtained were purified by mild physicochemical methods and the proteins purified with the proteoglycans were studied.

Experimental

Materials

Tracheal rings from adult cows were obtained directly from the slaughterhouse. The tracheal cartilage was dissected free from perichondrium and other surrounding tissue, frozen in liquid N_2 and ground in a Wiley mill. This procedure was completed within a few hours after slaughter. The ground cartilage was stored frozen at $-20^\circ C$ until used.

Sephacrose gels and protein standards for molecular-weight determination were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose (Whatman DE-52) was from W. and

Abbreviation used: SDS, sodium dodecyl sulphate.

R. Balston, Maidstone, Kent, U.K.; chondroitinase ABC (Seikagaku) was from Miles Laboratories, Elkhart, IN, U.S.A.; papain (type III) was from Sigma Chemical Co., St. Louis, MO, U.S.A. All chemicals used were of analytical grade.

Preparative procedure

Ground cartilage (10g) was suspended in 200ml of ice-cold 0.15M-NaCl/5mm-sodium phosphate/10mm-EDTA/0.1M-6-aminohexanoic acid/1mm-phenylmethanesulphonyl fluoride, pH 7.4, and homogenized for 30min at top speed in a Sorvall Omni-mixer. During homogenization the sample container was submerged in an ice/water mixture. The homogenate was clarified by centrifugation for 30min at 25000g_{av.} at 4°C. The pellet was suspended in 200ml of 4M-guanidinium chloride/5mm-sodium phosphate/10mm-EDTA/0.1M-6-aminohexanoic acid/1mm-phenylmethanesulphonyl fluoride, pH 7.4, and stirred at 4°C for 20h. Unextractable material was recovered by centrifugation as above, extensively dialysed against distilled water and freeze-dried. In a control experiment the homogenization was omitted and the cartilage was directly extracted with 4M-guanidinium chloride containing the proteinase inhibitors. In all instances, portions of homogenate and extracts not immediately used were frozen and not thawed until further processed.

Analytical methods

Hexuronic acid and protein. Hexuronic acid content in column effluents was determined by an automated version (Heinegård, 1973) of the carbazole procedure (Bitter & Muir, 1962). Protein was determined as the absorbance of solutions at 280nm.

Chondroitin sulphate. Chondroitin sulphate content was determined after papain digestion of samples in 0.05M-sodium phosphate/5mm-EDTA/5mm-cysteine hydrochloride, pH 6.5. Approx. 2μl of a crystalline papain suspension (25mg of protein/ml) was used per mg of material. Samples were digested for 72h at 60°C and then applied to an ion-exchange DEAE-cellulose column, which was eluted with 0.02M-HCl and 6M-HCl. The 6M-HCl eluate contained the chondroitin sulphate and was immediately evaporated to dryness. These samples were then hydrolysed in 4M-HCl under argon at 100°C for 10h. The content of galactosamine in the hydrolysates was determined by using an automatic amino acid analyser and taken as a measure of chondroitin sulphate content.

Gel chromatography. Sepharose CL-2B columns (0.8cm×150cm) were eluted at 4°C with 0.15M-NaCl/5mm-sodium phosphate, pH 7.4 (associative buffer), and 4M-guanidinium chloride/5mm-sodium phosphate, pH 7.4 (dissociative buffer), respectively. Fractions of volume about 1.2ml were collected.

Sucrose-density-gradient centrifugation. A 1ml

portion of homogenate was layered on a continuous gradient (10ml) of 10–50% (w/v) sucrose in the associative buffer. The sample was centrifuged for 6h at 38000 rev./min (180000 g_{av.}) at 18°C in an MSE SS65 ultracentrifuge with the 6×14ml titanium swing-out rotor. Fractions of volume 1ml were collected from the bottom of the tubes by using an LKB Varioprep peristaltic pump and a drop counter. Before analysis the sucrose was removed by extensive dialysis against water.

Ion-exchange chromatography. A column (10ml packed volume) of DEAE-cellulose was equilibrated with the starting buffer (0.15M-NaCl/5mm-sodium phosphate, pH 7.4). The sample (1ml of cartilage homogenate) was applied and the column was eluted with 20ml of starting buffer at 4°C. Elution was continued with a linear gradient of 0.15–1.0M-NaCl/5mm-sodium phosphate, pH 7.4, the total volume being 100ml. Fractions of volume approx. 2.25ml were collected.

Gel electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed on 8% acrylamide gels essentially as described by Neville (1971). The samples had to be pretreated with chondroitinase ABC as follows. Freeze-dried samples were dissolved in 0.1M-Tris/0.1M-sodium acetate, pH 7.3. Chondroitinase ABC (4munits/mg of sample) was added and the digestion mixtures were incubated at 37°C for 6h. The digests were then dialysed against distilled water, freeze-dried, dissolved in twice-concentrated upper-reservoir buffer supplemented with 10% (w/v) sucrose and 0.01% EDTA (when reducing conditions were desired also containing 10% 2-mercaptoethanol) and incubated at 37°C for 2h before electrophoresis. The gels were stained with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.). Phosphorylase b (mol.wt. 94000), serum albumin (mol.wt. 67000), catalase subunit (mol.wt. 60000), ovalbumin (mol.wt. 43000), lactate dehydrogenase subunit (mol.wt. 36000), carbonic anhydrase (mol.wt. 30000) and soya-bean trypsin inhibitor (mol.wt. 20100) were used as standards for molecular-weight determination.

Results

Preparation

In the preparative procedure, two soluble fractions, the homogenate and the subsequent guanidinium chloride extract, were obtained. The distribution of chondroitin sulphate between the extracts and the residue was determined as described in the Experimental section (Table 1). For comparison the corresponding results from a direct extraction with 4M-guanidinium chloride with omission of the homogenization step are given. Almost half of the tissue proteoglycans were recovered in the homogenate and another 48% were extracted with 4M-

Table 1. *Distribution of chondroitin sulphate in extracts and residue*

Experimental details are given in the text. Chondroitin sulphate was determined as galactosamine and the distribution expressed as percentage of total recovered tissue galactosamine.

	Distribution of chondroitin sulphate (%)	
	Homogenization followed by guanidine extraction	Guanidine extraction
Homogenate	46.1	
Guanidine extract	47.5	95.0
Residue	6.4	5.0

guanidinium chloride. Only 6.4% of the proteoglycans remained in the residue.

Portions of the homogenate and the subsequent guanidine extract were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 1). In this system most of the proteins of the samples were resolved. The proteoglycans are, however, excluded from the gels and remain at the top of the spacer gel, even after chondroitinase ABC treatment (Fig. 1). In the top portion of the gels one or two bands, probably representing small amounts of α -chains of collagen, can be seen. The most intensely stained band migrates with an apparent molecular weight of 60000. This protein was designated cartilage matrix protein. It was present both in the homogenate and in the subsequent guanidine extract. When less sample was applied it was resolved into two bands, with apparent mol.wts. of 61000 and 59000. The link proteins were also detected in both extracts. Link protein *a* has an apparent mol.wt. of 49500 and link protein *b* one of 45500. Occasionally a faint band was observed at the position corresponding to a mol.wt. of 43500. This component probably represents a subfraction of link protein *b* and may correspond to the protein that Baker & Caterson (1977) suggested to be a third link protein. Three protein bands with mol.wts. of 25000–35000 were observed. At least one of them (mol.wt. 35000) was always present in homogenates and extracts.

The homogenate and the subsequent guanidine extract were also analysed by SDS/polyacrylamide-gel electrophoresis without prior treatment with 2-mercaptoethanol (Fig. 1). A high-molecular-weight protein, not present in the reduced samples, appeared near the top of the gels. Very little protein, on the other hand, migrated to the position of the reduced cartilage matrix protein. Therefore it appears that the intact cartilage matrix protein has a mol.wt. of more than 200000 and may be dissociated to yield subunits when disulphide bridges are reduced. Another component migrated with a somewhat higher

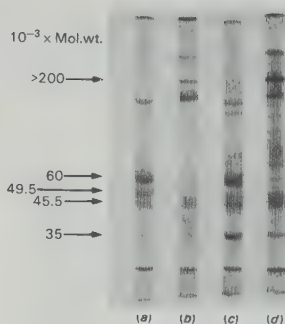


Fig. 1. *SDS/polyacrylamide-gel electrophoresis of extracts of tracheal cartilage*

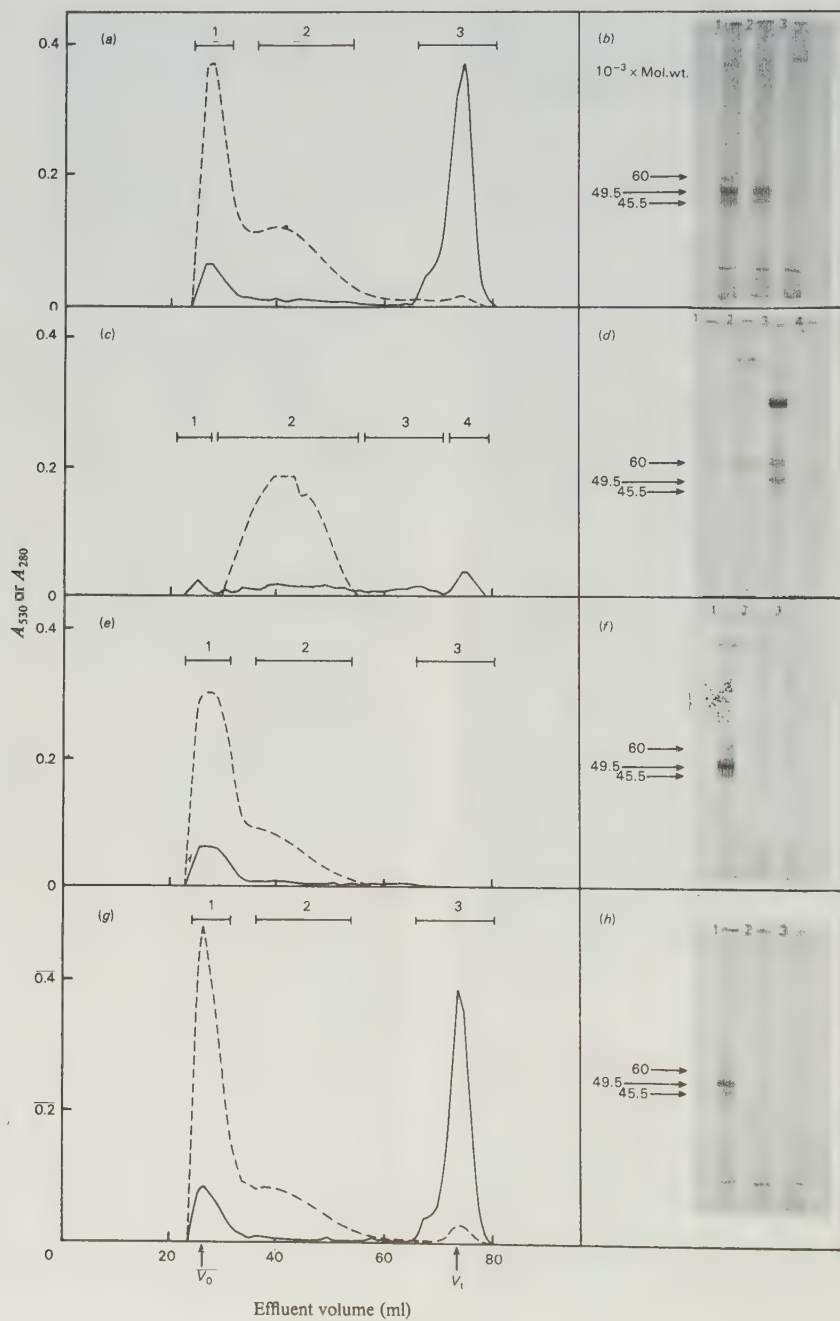
Experimental details are given in the text. (a) Homogenate treated with 2-mercaptoethanol; (b) identical sample not treated with 2-mercaptoethanol. (c) 4M-Guanidinium chloride extract of homogenization residue treated with 2-mercaptoethanol; (d) identical sample not treated with 2-mercaptoethanol.

mobility to the position of collagen α -chains with an apparent mol.wt. of about 130000. Some protein did not penetrate the separation gel when the samples were not reduced. This protein may contribute to the bands observed at the position of the matrix protein subunits with the reduced samples.

Gel chromatography

The SDS/polyacrylamide-gel electrophoresis of the homogenate revealed the existence of several tissue proteins in addition to the link proteins. Therefore it was relevant to determine whether these proteins could interact with the proteoglycans. For this purpose, freshly prepared homogenate (1 ml) was fractionated by sucrose-density-gradient centrifugation, ion-exchange chromatography and gel chromatography. One portion was chromatographed on Sepharose CL-2B in the associative buffer. The proteoglycans were eluted in a typical pattern with an excluded peak of aggregates and an included peak of monomers (Heinegård, 1972) (Fig. 2a). Only very small amounts of uronic acid could be detected being eluted late in the chromatogram, indicating that degradation during homogenization was negligible. The peak of u.v.-absorbing material at the total volume of the column was at least partly due to the proteinase inhibitors present in the homogenization buffer. The fractions were pooled as indicated in Fig. 2(a), dialysed against distilled water and freeze-dried.

Samples representing an equal proportion of the material in each pool were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 2b). Both the link proteins and the cartilage



matrix protein were observed in both the aggregate and in the monomer fractions. Only small amounts of protein were detected in the third pool containing molecules of low molecular weight. The results show that not only the link proteins but also the cartilage matrix protein chromatograph with the larger proteoglycans, indicating an interaction.

A sample of the homogenate was transferred to dissociative conditions by dialysis against 4M-guanidinium chloride/5mM-sodium phosphate, pH 7.4, and then chromatographed on a Sepharose CL-2B column eluted with 4M-guanidinium chloride (Fig. 2c). The fractions were pooled as indicated. The SDS/polyacrylamide-gel electrophoresis (Fig. 2d) of samples representing identical proportions of the pools showed that most of the cartilage matrix protein was eluted in a peak distinct from and more retarded than the proteoglycans. It appears, then, that the interaction of the matrix protein with the proteoglycans is broken by 4M-guanidinium chloride.

To determine whether the dissociation was reversible, another sample of the homogenate was first dissociated as described above and then dialysed back into the associative buffer. When chromatographed on Sepharose CL-2B in the associative buffer it showed a uronic acid profile similar to that of the untreated homogenate (Fig. 2e). SDS/polyacrylamide-gel electrophoretic analysis of identical samples of the pools demonstrated that most of the cartilage matrix protein was eluted with the proteoglycans, indicating that the protein could again interact when the dissociating solvent was removed (Fig. 2f).

To determine whether the homogenate contained endogenous proteinases that were not inhibited by the proteinase inhibitors added, a sample was left at room temperature for 48h before chromatography on Sepharose CL-2B in the associative buffer (Fig. 2g). No additional uronic acid-containing peak that was eluted late was observed, indicating that the proteoglycans were not degraded during incubation. Indeed the proportion of excluded material increased,

indicating that a larger amount of proteoglycan monomers was included in aggregates. SDS/polyacrylamide-gel electrophoretic analysis revealed that virtually all of the cartilage matrix protein and the link

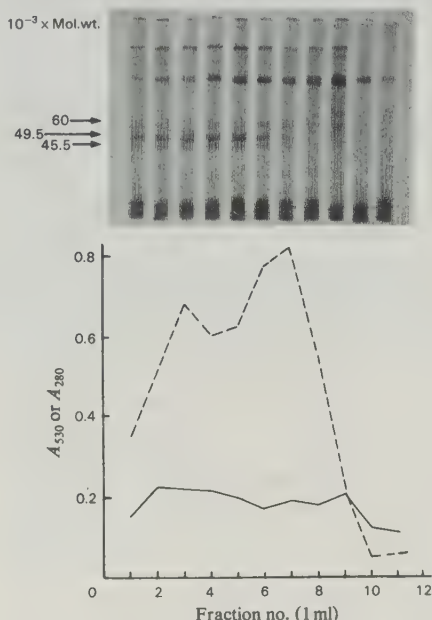


Fig. 3. Sucrose-density-gradient centrifugation of homogenate of tracheal cartilage

A 1ml sample of homogenate was subjected to sucrose-density-gradient centrifugation as described in the text. The fractions were analysed by SDS/polyacrylamide-gel electrophoresis after treatment with 2-mercaptoethanol. ----, A_{330} (carbazole reaction); —, A_{280} (protein).

Fig. 2. Sepharose CL-2B chromatography of cartilage homogenates

Experimental details are given in the text. Sepharose CL-2B gel chromatography of homogenate is shown in (a). The column was eluted with the associative buffer. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (b) after treatment with 2-mercaptoethanol. Sepharose CL-2B gel chromatography of homogenate transferred to the dissociative buffer by dialysis is shown in (c). The column was eluted with the dissociative buffer. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (d) after treatment with 2-mercaptoethanol. Sepharose CL-2B gel chromatography of homogenate first dissociated by dialysis against the dissociative buffer and then reassociated by dialysis against the associative buffer is shown in (e). The column was eluted with the associative buffer. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (f) after treatment with 2-mercaptoethanol. Sepharose CL-2B gel chromatography of homogenate incubated at room temperature for 48h before chromatography is shown in (g). The column was eluted with the associative buffer. The fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis (h) after treatment with 2-mercaptoethanol. Gels of the pools are shown with increasing number from left to right. ----, A_{330} (carbazole reaction); —, A_{280} (protein). V_0 , Void volume; V_t , total volume.

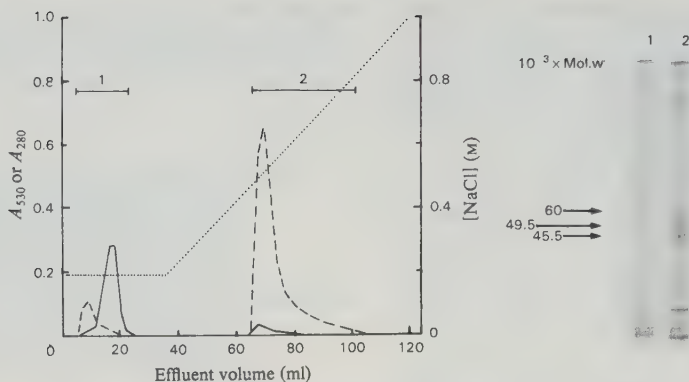


Fig. 4. DEAE-cellulose chromatography of homogenate of tracheal cartilage

A 1 ml sample of homogenate was subjected to DEAE-cellulose chromatography as described in the text. The column (packed volume 10 ml) was first eluted with 20 ml of 0.15 M-NaCl/5 mM-sodium phosphate, pH 7.4, and then with a linear gradient of 0.15–1.0 M-NaCl/5 mM-sodium phosphate, pH 7.4. Fractions were pooled as indicated by the bars and analysed by SDS/polyacrylamide-gel electrophoresis after treatment with 2-mercaptoethanol. ---, A_{530} (carbazole reaction); —, A_{280} (protein); ·····, concn. of NaCl.

proteins were eluted in the aggregate fraction (Fig. 2h). The lower proportion of aggregates in the homogenate may be due to a limited disruption of aggregates during homogenization, liberating proteoglycan monomers that were capable of forming aggregates when incubated. Another possibility is that a fraction of the proteoglycans in the tissue are unable to bind to hyaluronic acid for steric reasons. When the collagen network is disrupted during homogenization they are no longer hindered and may eventually bind to hyaluronic acid.

Sucrose-density-gradient centrifugation

Another method used to fractionate the components of the homogenate was sucrose-density-gradient centrifugation. The proteoglycans were distributed in a large number of fractions (Fig. 3). The faster-sedimenting aggregates can, however, be distinguished from more slowly sedimenting proteoglycan monomers. The absorbance at 280 nm shows a minor peak at the position of the aggregates. The content of various proteins in the fractions was studied by SDS/polyacrylamide-gel electrophoresis (Fig. 3). The cartilage matrix protein co-distributed with both the proteoglycan aggregates and the proteoglycan monomers, whereas the link proteins were predominantly present in the fractions containing aggregates. Some collagen was found even in the rapidly sedimenting fractions. Whether this was due to collagen–proteoglycan interactions was not further studied.

Ion-exchange chromatography

Both gel chromatography and sucrose-density-gradient centrifugation separate molecules according to parameters related to their weight and shape. Therefore it was possible that the co-fractionation of the cartilage matrix protein with the proteoglycans was due to the formation of high-molecular-weight homoaggregates of this protein. On ion-exchange chromatography, however, proteins are eluted at a lower ionic strength than are the highly charged proteoglycans, unless the components are associated by salt-resistant bonds. DEAE-cellulose chromatography resolved the homogenate into two fractions (Fig. 4). The cartilage matrix protein was eluted with the link proteins and the proteoglycans, late in the gradient (Fig. 4), indicating that these components form complexes. This further supports the existence of an interaction between the cartilage matrix protein and the proteoglycans. Furthermore, the interaction seems to be stable also at higher concentrations of NaCl.

Discussion

The choice of gel chromatography and sucrose-density-gradient centrifugation as means of fractionating the cartilage homogenate was determined by a desire to isolate proteoglycans in an ionic environment as close to the physiological one as possible, thereby preserving even weak interactions with other structural components. By this approach a previously

unrecognized protein, present both in tracheal and nasal cartilage (M. Paulsson & D. Heinegård, unpublished work), could be shown to co-fractionate with the proteoglycans in both fractionation procedures. This protein, the cartilage matrix protein, has a high molecular weight and contains what seem to be two different kinds of subunits (with apparent mol.-wts. of 61000 and 59000) probably joined by disulphide bonds. The stoichiometry of the complex is not yet known. Currently it cannot be precluded that the matrix protein represents more than one protein component not resolved by the techniques used. Only one or two different protein subunits were, however, observed on reduction. The presently available data indicate that the cartilage matrix protein interacts with the proteoglycan monomers as well as with the aggregates. The fact that the protein remains bound to the proteoglycans during ion-exchange chromatography when the ionic strength is substantially increased implies that the interaction is relatively stable. The high concentration of salt used in the conventional CsCl-density-gradient centrifugation, however, breaks the interaction, even though small amounts of cartilage matrix protein can occasionally be detected in the A1 fraction. Most previous investigations of cartilage proteins have been focused on proteins that can be dissociated from the A1 fraction. Therefore the cartilage matrix protein has escaped our attention until recently.

Homogenates and extracts of cartilage also contain other proteins, which, even if they interact with proteoglycans to a smaller extent, still might contribute to stabilizing the proteoglycan aggregates and the proteoglycan-collagen interactions. It is

likely that the characteristic properties of cartilage, such as elasticity, are to a large extent the result of multiple interactions between the molecular constituents of the tissue.

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Purification and structural characterization of a cartilage matrix protein

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The cartilage matrix protein is a major non-collagenous protein in bovine cartilage. It was purified from a 5 M-guanidinium chloride extract of bovine tracheal cartilage by sequential CsCl-density-gradient centrifugation, gel chromatography in guanidinium chloride and differential precipitation. The molecular weight of the intact protein is 148 000, determined by sedimentation-equilibrium centrifugation. It was dissociated to three subunits of molecular weight 52 000 by reduction of disulphide bonds. The cartilage matrix protein was insoluble in low-salt solutions and behaved abnormally on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The content of cysteine was high, whereas the contents of aromatic amino acids were low. The carbohydrate content was 3.9% (w/w). Glycopeptides obtained after papain digestion were heterogeneous on gel chromatography. Asparagine/aspartic acid was enriched in the purified glycopeptides, indicating the presence of *N*-glycosidic linkages to protein.

Physical properties of cartilage depend on the molecular characteristics of its major constituents, collagen and proteoglycans. Probably even more important is the molecular organization of the tissue. Multiple intermolecular interactions, some of which have been characterized (Hardingham & Muir, 1972; Heinegård & Hascall, 1974; Caterson & Baker, 1978), are probably essential for the three-dimensional structure of the tissue matrix.

The cartilage matrix protein was first identified as a component in cartilage proteoglycan preparations that had been extracted by high-speed homogenization in a low-salt buffer and purified by mild techniques (Paulsson & Heinegård, 1979). It could be separated from proteoglycans when transferred to 4 M-guanidinium chloride. It was also extracted in good yield from cartilage by 4 M-guanidinium chloride.

There are relatively few non-collagenous proteins in cartilage. In both bovine tracheal and nasal cartilages the cartilage matrix protein is one of the quantitatively dominating proteins. Another major protein, the link protein, important in proteoglycan aggregate formation, is the only non-collagenous protein that has been studied in some detail (Baker & Caterson, 1979; Périn *et al.*, 1980). The object of the present study was to purify and structurally characterize the cartilage matrix protein, as part of the overall aim of understanding the function and organization of the cartilage matrix.

Experimental

Materials

Bovine tracheal rings from young animals were obtained directly from the slaughterhouse. The tracheal cartilage was freed from surrounding tissue and perichondrium, frozen in liquid N₂ and ground frozen in a Wiley mill. This procedure was completed within a few hours after slaughter. The ground cartilage was stored frozen at –20°C until used.

Preparation of cartilage matrix protein

Extraction. A 20 g portion of ground cartilage was suspended in 200 ml of ice-cold 5 M-guanidinium chloride/5 mM-sodium phosphate buffer, pH 7.4, containing 0.1 M-6-aminohexanoic acid, 10 mM-EDTA and 10 mM-*N*-ethylmaleimide. The extraction was allowed to proceed for 20 h at 4°C under continuous stirring. Unextracted material was removed by centrifugation at 25 000 *g*_{av} for 30 min.

CsCl-density-gradient centrifugation. The extract was adjusted to a density of 1.40 g/ml by addition of solid CsCl. It was then centrifuged at 35 000 rev./min (95 000 *g*_{av}, 6.886 cm) for 48 h at 18°C in the MSE 8 × 25 ml aluminium angle rotor. Tubes were frozen by immersion in liquid N₂ and cut in three parts of equal volume, while still frozen. The top fraction was dialysed overnight at 4°C against 20 vol. of 4 M-guanidinium chloride/5 mM-sodium

phosphate buffer, pH 7.4, to remove most of the CsCl.

Gel chromatography. Portions (20 ml) of the top fraction from the CsCl gradient were chromatographed on a column (3.0 cm × 145 cm) of Sephadex G-200 eluted with 4 M-guanidinium chloride/5 mM-sodium phosphate buffer, pH 7.4. Fractions of volume 10 ml were collected. Fractions (pool 1, Fig. 2a) from two chromatograms were pooled and concentrated to 20 ml by ultrafiltration (Amicon PM-10, cut-off mol.wt. 10000). The sample was chromatographed on a column (3.0 cm × 145 cm) of Sepharose CL-6B. Elution and fraction collection was done as described above.

Precipitation. The cartilage matrix protein was precipitated after Sepharose CL-6B chromatography. Pool 3 (Fig. 3a) was dialysed against 39 vol. of 5 mM-sodium phosphate buffer, pH 7.4, for 48 h, so that a concentration of 0.1 M-guanidinium chloride was reached. The precipitate was collected by centrifugation at 25000 g_{av} for 30 min and washed by suspension in distilled water and re-centrifugation in cycles. The supernatant was dialysed against another 39 vol. of the phosphate buffer, and the precipitate formed was recovered as described above. All procedures were at 4°C. The final supernatant was extensively dialysed against distilled water and freeze-dried.

Preparation of subunits

A 15 mg portion of cartilage matrix protein was dissolved in 5 ml of 6 M-guanidinium chloride/0.10 M-Tris/HCl buffer, pH 8.0. Dithiothreitol was added to a concentration of 10 mM and the sample was incubated at 37°C for 5 h. The reduced protein was alkylated by addition of iodoacetic acid to a final concentration of 30 mM and incubation overnight in darkness. The sample was diluted to 4 M-guanidinium chloride by addition of 2.5 ml of distilled water and chromatographed on a column (3.0 cm × 145 cm) of Sephadex G-200 eluted with 4 M-guanidinium chloride/5 mM-sodium phosphate buffer, pH 7.4. Fractions containing subunits were pooled and the material was recovered by dialysis and freeze-drying.

Preparation of glycopeptides

Papain digestion. A 10.2 mg portion of cartilage matrix protein was suspended in 1 ml of 0.1 M-sodium phosphate buffer, pH 6.5, containing 5 mM-EDTA and 5 mM-dithiothreitol. Then 4 μ l of papain (type III; Sigma Chemical Co., St. Louis, MO, U.S.A.) (enzyme/substrate ratio 1:100) was added and the sample was incubated at 60°C for 36 h. After 12 h and 24 h another 4 μ l of papain and 50 μ l of 0.1 M-dithiothreitol in the digestion buffer was added. The digest was chromatographed on a column (0.8 cm × 250 cm) of Bio-Gel P-10 eluted

with 0.5 M-pyridinium acetate buffer, pH 6.5. Fractions of volume 1.1 ml were collected.

Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Neville (1971). The bottom fraction from the CsCl-density-gradient centrifugation was pretreated with chondroitinase ABC (Paulsson & Heinegård, 1979) to avoid collapse of the stacking gel. Samples were reduced with 2-mercaptoethanol unless otherwise stated. Purification was followed by electrophoresis of samples representing equal proportions of each pool. This was done on gels 8.0 × 2.5%, where the first numeral (T) designates the total amount of monomer (% w/v) and the second numeral (C) designates the amount of NN'-methylenebisacrylamide, expressed as a fraction (% w/w) of total monomer in the gel casting solution. A Ferguson (1964) plot was obtained by comparison of the relative mobilities of the major subunit of the cartilage matrix protein with those of reference proteins (high-molecular-weight and low-molecular-weight standard proteins; Pharmacia Fine Chemicals, Uppsala, Sweden) on gels 5–11 × 2.5%. Gels were stained for protein with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.).

Analytical ultracentrifugation

Preparation of samples. All samples were dissolved in 6 M-guanidinium chloride (Ultrapure; Schwarz-Mann, Orangeburg, NY, U.S.A.)/5 mM-Tris/HCl buffer, pH 7.0. In all experiments a stock solution of the highest sample concentration was dialysed for 20–40 h against 25 vol. or more of the solvent. Weighed dilutions were made with the diffusate.

Determination of molecular weights. An MSE Centrican 75 analytical ultracentrifuge equipped with a photoelectric scanner and a 280 nm filter was used. Samples, giving 4 mm column height, were layered on fluorocarbon oil (FC 70; 3M, Minneapolis, MN, U.S.A.). High-speed equilibria were established essentially as described by Yphantis (1964). Samples with initial concentrations ranging from 0.1 mg/ml to 0.8 mg/ml were centrifuged at two or three different rotor speeds (cartilage matrix protein, 18000, 20000 and 22000 rev./min; cartilage-matrix-protein subunits, 28000 and 34000 rev./min) at 20°C.

Determination of sedimentation coefficients. Samples containing 0.8–3.2 mg of cartilage matrix protein or subunits/ml were centrifuged at 59000 rev./min at 20°C. The photoelectric scanner was used at 280 nm. The position of the boundary was determined as the r co-ordinate of the half-height of the tracing. The sedimentation was

followed to the bottom of the cell, corresponding to a minimum distance of 8 mm.

Determination of the apparent partial specific volume. The apparent partial specific volume of cartilage matrix protein in 6 M-guanidinium chloride was experimentally determined by the $^2\text{H}_2\text{O}$ method (Thomas & Edelstein, 1971) and calculated from the amino acid composition as described by Lee & Timasheff (1974). With both procedures a value of 0.730 ml/g was obtained.

Analytical procedures

Protein contents were determined as the absorbance of solutions at 280 nm.

Amino acid composition was determined by using an automatic amino acid analyser after hydrolysis in 6 M-HCl under argon at 110°C for 24 and 72 h. Cysteine and methionine were determined as cysteic acid and methionine sulphone respectively, after performic acid oxidation. Tryptophan was determined by the spectroscopic method of Edelhoch (1967).

Hexosamines were determined by using an automatic amino acid analyser after hydrolysis in 4 M-HCl under argon at 100°C for 10 h.

Hexuronic acid in column effluents was determined by an automated version (Heinegård, 1973) of the carbazole reaction (Bitter & Muir, 1962).

Analytical separations of neutral sugars were performed by a modification (S. Lohmander, personal communication) of the borate complex ion-exchange procedure of Walborg & Kondo (1970). Samples were hydrolysed in 2 M-trifluoroacetic acid for 3 h at 100°C. Neutral sugars in column effluents were determined by an automated version of the orcinol/ H_2SO_4 procedure, essentially as described by Kesler (1967).

Sialic acid was determined by the periodate/resorcinol procedure of Jourdain *et al.* (1971). When column effluents were analysed an automated version was used (Lohmander *et al.*, 1980).

Results

Preparative procedure

Proteoglycan contents and absorbance at 280 nm of the fractions from the CsCl-density-gradient-equilibrium centrifugation were determined (Fig. 1a). The distribution of proteins in the fractions was studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1b). The proteoglycans were almost quantitatively recovered in the bottom fraction, as indicated by the uronic acid. Most of the 280 nm absorbance detected in the bottom two fractions was due to the proteinase inhibitors and contaminations in the CsCl used. The top fraction contained very little uronic acid, but most of the non-proteoglycan protein could be

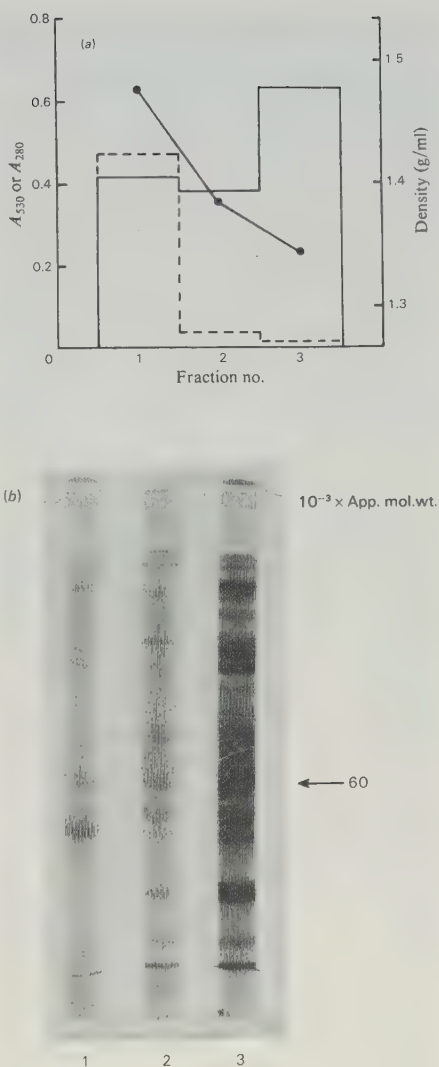


Fig. 1. (a) CsCl-density-gradient centrifugation of the cartilage extract and (b) sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the fractions obtained

Experimental details are given in the text. (a) The samples were diluted with 9 vol. of distilled water before the determination of uronic acid and protein contents. —, A_{430} (carbazole reaction); —, A_{280} ; ●—●, density (g/ml). (b) The sample from fraction 1 was digested with chondroitinase ABC to remove chondroitin sulphate chains before electrophoresis. All samples were treated with 2-mercaptoethanol.

recovered from this fraction. Among the proteins in this fraction, the subunits of the cartilage matrix protein showed as a prominent band with an apparent molecular weight of about 60 000.

Samples of the top fraction were chromatographed on a Sephadex G-200 column eluted with 4M-guanidinium chloride/5mM-sodium phosphate buffer, pH 7.4 (Fig. 2a). The protein was eluted in three major peaks. The middle peak contained more sialic acid, compared with the other peaks, indicating a higher oligosaccharide content. Fractions corresponding to the peaks were pooled as indicated, and samples were reduced and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 2b). The major portion of the cartilage matrix protein was present in the first pool and was eluted in or close to the void volume. This fraction also contained a number of other high-molecular-weight proteins. Some of these appeared to be of collagenous nature, as revealed by the reddish staining obtained with Kenacid Blue R (McCormick *et al.*, 1979). The second pool contained a predominant protein appearing as a broad diffuse band with an apparent molecular weight of 70 000–90 000. The electrophoretic mobility and polydispersity are similar to the behaviour of the hyaluronic acid-binding region fragment of cartilage proteoglycans, isolated by trypsin digestion of proteoglycan aggregates (Heinegård & Hascall, 1974). This material reacted strongly with an antiserum directed against the isolated hyaluronic acid-binding region (results not shown) and therefore appears to represent free hyaluronic acid-binding region, probably fragmented from the cartilage proteoglycans by the physiological proteolytic catabolism. Caputo *et al.* (1980) have isolated a molecule with similar electrophoretic mobility from the Swarm rat chondrosarcoma. The third pool contains two proteins with apparent molecular weights of 49 500 and 45 500, mobilities characteristic of the link proteins (Keiser *et al.*, 1972). A faint band present below the 45 500-mol.wt. link protein probably represents the third link protein, described by Baker & Caterson (1979). In addition, an intensely stained protein with an apparent molecular weight of 35 000 is present.

After concentration by ultrafiltration the first pool was rechromatographed on Sepharose CL-6B eluted with 4M-guanidinium chloride/5mM-sodium phosphate buffer, pH 7.4 (Fig. 3a). The chromatogram showed three distinct peaks and a leading shoulder on the intermediate peak. Fractions corresponding to the peaks were pooled and equal portions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 3b). The cartilage matrix protein was eluted in the third pool, together with a protein that stained like collagen. The first two pools contained high-molecular-weight material,

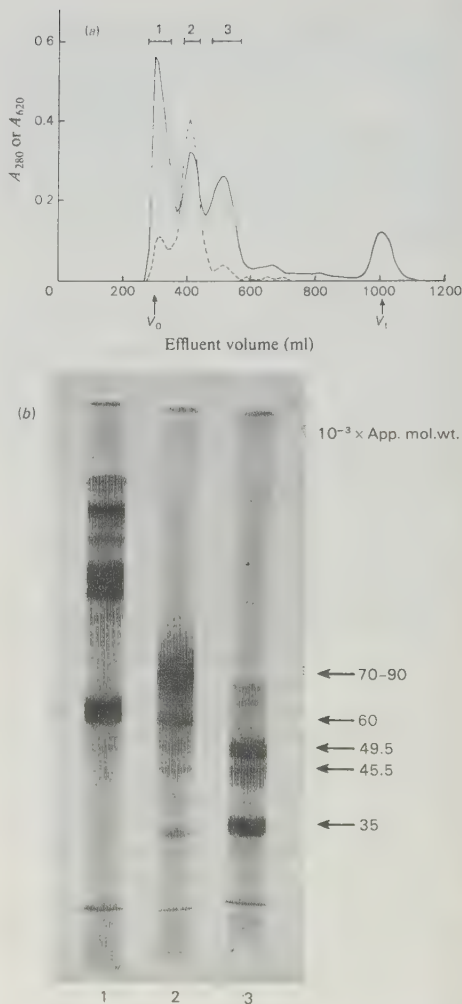


Fig. 2. (a) Sephadex G-200 gel chromatography of the top fraction from the CsCl-density-gradient centrifugation of the cartilage extract and (b) sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of pooled fractions obtained

Experimental details are given in the text. (a) A 20 ml portion of the top fraction (Fig. 1a) was dialysed against 4M-guanidinium chloride/5mM-sodium phosphate buffer, pH 7.4, and applied to the column. The chromatography was performed as described in the text. Fractions were pooled as indicated by the bars. —, A_{280} ; ---, A_{620} (sialic acid). V_0 , Void volume; V_t , total volume. (b) The samples correspond to the pools from the Sephadex G-200 chromatogram as indicated by numbers. The samples were treated with 2-mercaptoethanol before electrophoresis.

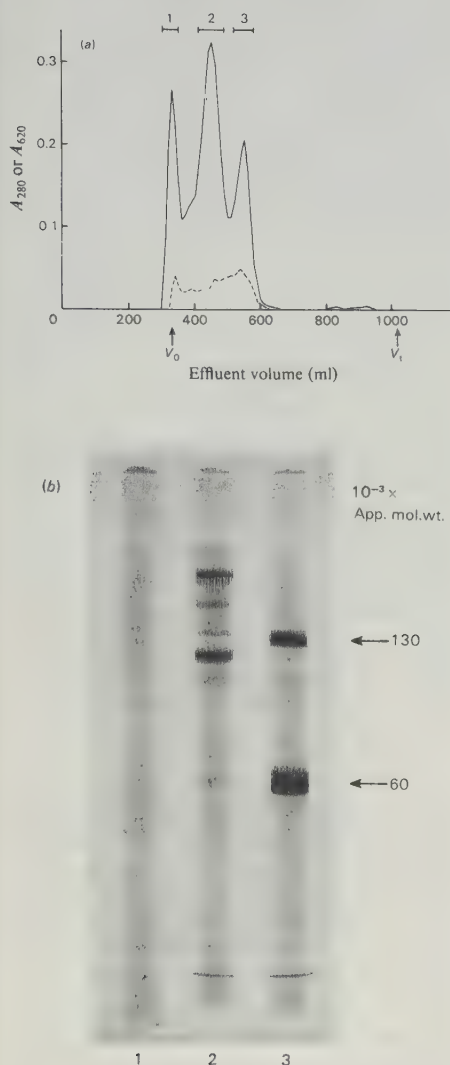


Fig. 3. (a) Sepharose CL-6B gel chromatography of the cartilage-matrix-protein-containing pool from the Sephadex G-200 chromatography and (b) sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of pooled fractions obtained

Experimental details are given in the text. (a) The first pools from two Sephadex G-200 chromatograms (Fig. 2a) were concentrated by ultrafiltration and applied to the column. Elution and fraction collection was as described in the text. Fractions were pooled as indicated by the bars. —, A_{280} ; ----, A_{620} (sialic acid). V_0 , Void volume; V_t , total volume. (b) The samples correspond to the pools from the Sepharose CL-6B chromato-

some of which did not enter the 8% polyacrylamide gels.

The two proteins present in the third pool of the Sepharose CL-6B chromatogram differed significantly in their solubility at lowered ionic strength. Most of the cartilage matrix protein was precipitated when the guanidinium chloride concentration was lowered to 0.1M by dialysis, whereas all of the other protein remained in solution (Fig. 4a). The small amount of precipitate formed when the guanidinium chloride concentration was lowered to 2.5 mM was a mixture of the two proteins (Fig. 4b). The supernatant contained virtually only the other protein (Fig. 4c). After being desalted the fractions were freeze-dried. Approx. 17mg of the first precipitate, 3mg of the second precipitate and 29mg of soluble protein were obtained per 10g of cartilage wet weight.

Homogeneity

The purity of the preparation of cartilage matrix protein was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on gels $7 \times 2.5\%$ and Sepharose CL-6B gel chromatography in 1% sodium dodecyl sulphate/50 mM-sodium bicarbonate buffer, pH 7.5 (results not shown). On electrophoresis the protein migrated as an apparently homogeneous band and could be quantitatively shifted to the position of the subunits when reduced, which was taken as a criterion of homogeneity. On visual inspection of the stained gel the subunits displayed some microheterogeneity. Two very closely spaced bands could be seen. The more intensely staining band had the higher mobility. These findings show that the cartilage matrix protein is an oligomer, most probably disulphide-bonded. Both the intact and the reduced matrix protein were eluted as single peaks on Sepharose CL-6B chromatography in the presence of sodium dodecyl sulphate (results not shown). Neither low-molecular-weight nor high-molecular-weight contaminants were present.

Electrophoretic behaviour

In previous work (Paulsson & Heinegård, 1979) we have used sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on gels $8 \times 2.5\%$ to assign apparent molecular weights to the subunits of the cartilage matrix protein. In the present study it was found that the apparent molecular weights could vary considerably with acrylamide concentration.

gram as indicated by numbers. The samples were treated with 2-mercaptoethanol before electrophoresis.

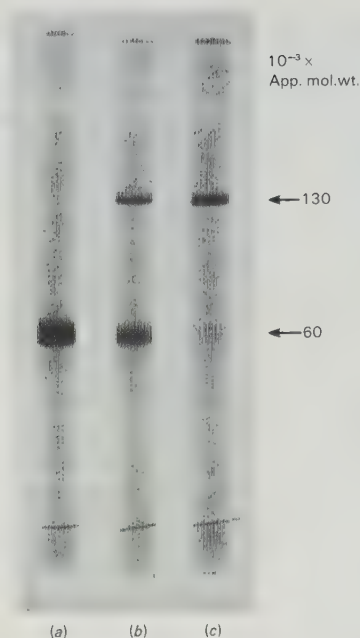


Fig. 4. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fractions obtained by differential precipitation of the cartilage matrix protein

Experimental details are given in the text. The samples were treated with 2-mercaptoethanol before electrophoresis. (a) First precipitate; (b) second precipitate; (c) soluble protein.

As a control, a Ferguson (1964) plot of $\log R_F$ versus the gel concentration was constructed for the major subunit of the cartilage matrix protein and for a variety of reference proteins (Fig. 5). The relative mobility of the matrix protein is not solely a function of its molecular weight, since the plot does not extrapolate to the point of convergence for the reference proteins. The major subunit of the cartilage matrix protein forms a sodium dodecyl sulphate complex of higher apparent free electrophoretic mobility than the reference proteins used (Fig. 5). It is possible that the cartilage matrix protein binds sodium dodecyl sulphate in excess of the 1.4 g/g bound by most proteins (Reynolds & Tanford, 1970). It should be stressed that the abnormal migration on electrophoresis invalidates attempts to determine molecular weights of the cartilage matrix protein subunits by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Determination of molecular weight

Results obtained from several sedimentation-

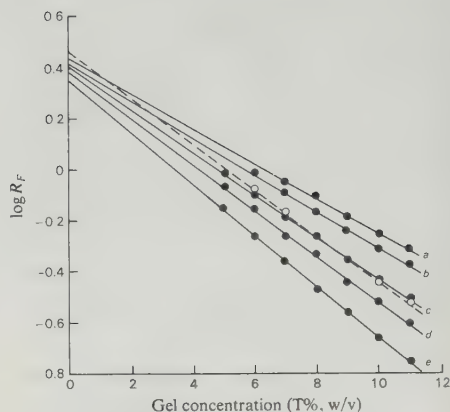


Fig. 5. Ferguson plot of $\log R_F$ versus gel concentration (T%) obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the subunits of the cartilage matrix protein and reference proteins

Experimental details are given in the text. For each electrophoretic run approx. 1 μ g of each protein was used. All samples were treated with 2-mercaptoethanol before electrophoresis. Curve a, lactate dehydrogenase subunit (mol.wt. 36 000); curve b, ovalbumin (mol.wt. 43 000); curve c, catalase subunit (mol.wt. 60 000); curve d, serum albumin (mol.wt. 67 000); curve e, phosphorylase b (mol.wt. 94 000). $\bigcirc$ — $\bigcirc$, Cartilage matrix protein subunits.

equilibrium centrifugations are summarized in Fig. 6. By extrapolation to zero concentration the molecular weight of the cartilage matrix protein was determined to be 148 000 (± 2000) and that of the subunits to be 52 000 (± 2000). The apparent molecular weights exhibit a negative concentration-dependence, more markedly at higher speeds. This is expected, as the actual protein concentration at equilibrium is then higher. Plots of $\ln c$ versus r^2 were linear, indicating the absence of polydispersity and aggregation.

Determination of sedimentation coefficients

Results from velocity sedimentations of cartilage matrix protein and its subunits are depicted in Fig. 7. The values of $s_{20,w}^0$ were 4.41S and 2.01S respectively. The values of $s_{20,w}$ exhibit a negative concentration-dependence.

Protein composition

The amino acid composition (Table 1) was calculated from results obtained by hydrolysis for 24 and 72 h. The recoveries of serine and threonine were extrapolated to zero time. For other amino acids the highest value of the two was chosen. The cartilage

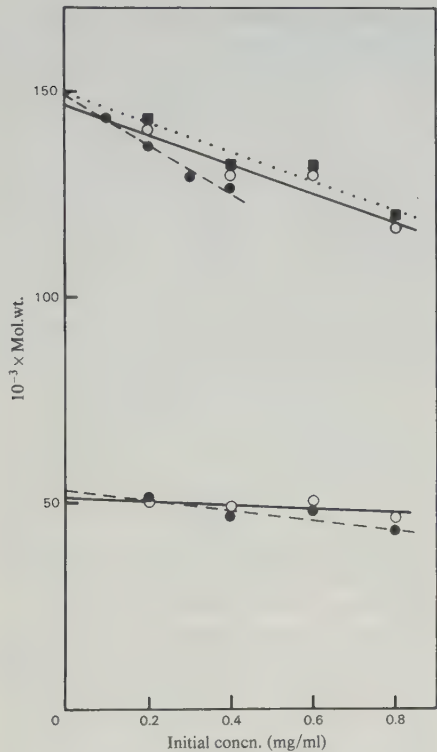


Fig. 6. Sedimentation-equilibrium centrifugation of the cartilage matrix protein and its subunits
Experimental details are given in the text. Upper part (cartilage matrix protein): ●—●, 22 000 rev./min; ■—■, 20 000 rev./min; ○—○, 18 000 rev./min. Lower part (cartilage matrix protein subunits): ●—●, 34 000 rev./min; ○—○, 28 000 rev./min.

matrix protein was found to be enriched in glutamic acid/glutamine and aspartic acid/asparagine. The contents of tryptophan and tyrosine are low and that of phenylalanine is moderate, as is also indicated by the low molar u.v. absorbance. The cysteine content is relatively high. Hydroxyproline could not be detected, indicating that cartilage matrix protein is non-collagenous.

Carbohydrate composition

Attempts to determine hexosamine contents in hydrolysates of the intact protein were unsuccessful, as the relatively small amounts of hexosamine were incompletely separated from non-hydrolysed peptides. Therefore cartilage matrix protein was digested with papain, and the glycopeptides were purified by

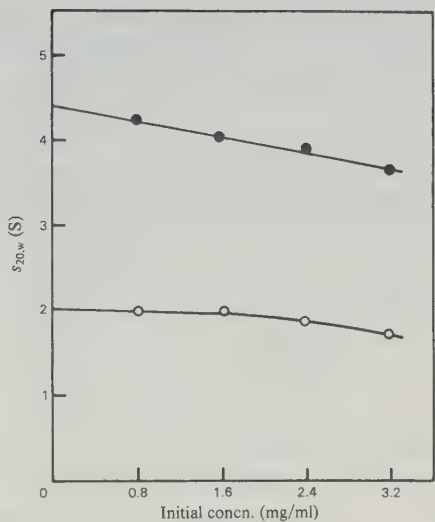


Fig. 7. Sedimentation-velocity centrifugation of the cartilage matrix protein and its subunits
Experimental details are given in the text. ●, Cartilage matrix protein; ○, cartilage matrix protein subunits.

Table 1. Amino acid composition of the cartilage matrix protein
Experimental details are given in the text.

Composition (residues/1000 residues)	
Asx	92.8
Thr	47.5
Ser	82.5
Glx	112.1
Pro	36.3
Gly	77.5
Ala	83.9
Cys	26.6
Val	88.2
Met	13.9
Ile	45.9
Leu	82.4
Tyr	19.1
Phe	39.5
His	20.0
Lys	63.7
Arg	65.3
Trp	2.7
Hyp	Not detected

Bio-Gel P-10 gel chromatography (Fig. 8). The heterogeneous population of glycopeptides was eluted in the included volume of the column. Two components were partially resolved. They were

Table 2. Carbohydrate composition of the cartilage matrix protein
Experimental details are given in the text.

	Composition			
	Intact protein		Glycopeptides	
	(nmol/mg)	(% of total)	(nmol/mg original wt.)	(% of total)
Amino acids*	7906.1	100.0	647.0	100.0
Asx	767.7	9.7	115.5	17.8
Ser	682.0	8.6	50.8	7.9
Thr	392.8	5.0	38.9	6.0
GlcN			85.5	
GalN			34.1	
Man			74.1	
Gal			9.5	
Fuc			15.3	
Xyl			<0.4	
Glc			Trace	
AcNeu			Not detected	

* The data do not take into account the contents of cysteine, methionine and tryptophan.

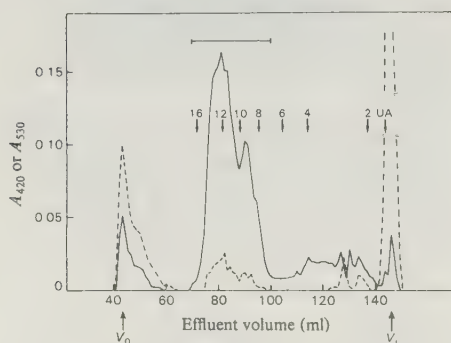


Fig. 8. Bio-Gel P-10 gel chromatography of the papain digest of the cartilage matrix protein

Experimental details are given in the text. The column was calibrated with hyaluronic acid oligosaccharides (Hascall & Heinegård, 1974) containing the numbers of monosaccharide residues indicated in the Figure. UA indicates the elution position of glucuronic acid. The carbazole peak at V_1 is due to the dithiothreitol present in the sample. The glycopeptides were pooled as indicated by the bar. —, A_{420} (orcinol reaction); ----, A_{530} (carbazole reaction). V_0 , Void volume; V_1 , total volume.

pooled as indicated, recovered in a quantitative manner and used for quantitative determination of hexosamine and neutral sugar. The material that was eluted in the void volume of the column reacted in the carbazole procedure. It probably represents glycosaminoglycans from a small contamination (1–2%, w/w) of proteoglycans in the preparation.

The contents of sugars and of relevant amino acids in the glycopeptides are given in Table 2. The total carbohydrate content of cartilage matrix protein was 3.9% (w/w). The predominating carbohydrates are glucosamine and mannose. This, taken together with the fact that aspartic acid/asparagine is markedly enriched in the glycopeptides, indicates that a major proportion of the oligosaccharides probably are of the high-mannose type and are bound to protein by an *N*-glycosidic linkage between glucosamine and asparagine (Kornfeld & Kornfeld, 1980). There is, however, a considerable amount of galactosamine present, a sugar not normally found in this type of oligosaccharide.

Discussion

Structural proteins of the connective-tissue matrix interact with various other tissue components. For the detailed characterization of such interactions the participating molecules are needed in pure form. The insolubility of many of the matrix molecules makes their purification and characterization difficult and often necessitates the use of chaotropic solvents. Some of the matrix molecules, e.g. the proteoglycans, the link proteins and presumably the cartilage matrix protein (Paulsson & Heinegård, 1979), do, however, recover their ability to interact when renatured. Therefore it is possible to extract the molecules with guanidinium chloride, purify them with the use of chaotropic solvents and then study their interactions when renatured.

The early attempts to purify the cartilage matrix protein were hampered by pronounced non-specific aggregation of proteins. This aggregation could be

prevented by including 10 mM-*N*-ethylmaleimide, to block free thiol groups, in the extraction buffer. It is therefore probable that the aggregation was due to disulphide exchange between proteins in the denatured state (Tanford, 1961).

The cartilage matrix protein is a non-collagenous protein consisting of polypeptide chains that migrate as two closely spaced bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Uneven distribution of the oligosaccharides among the protein subunits, however, would easily explain the microheterogeneity observed. Intact disulphide bonds are essential for the oligomeric structure. The molecular weights obtained for the cartilage matrix protein and its subunits, 148 000 (± 2000) and 52 000 (± 2000) respectively, are consistent with a trimeric structure of the intact protein.

It is as yet difficult to envisage the exact role of the cartilage matrix protein in the function of the tissue. From recoveries of purified protein, it can be estimated to constitute 1–2% of the tissue dry weight. This, taken together with its marked tendency to co-fractionate with proteoglycans (Paulsson & Heinegård, 1979), indicates that it is an integral part of the cartilage extracellular matrix.

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Radioimmunoassay of the 148-kilodalton cartilage protein

Distribution of the protein among bovine tissues

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A sensitive and specific radioimmunoassay for the 148 kDa cartilage protein (previously referred to as 'cartilage matrix protein') was developed. The protein is insoluble in conventional buffers and was therefore dissolved in sodium dodecyl sulphate. Excess sodium dodecyl sulphate was bound in mixed micelles with Triton X-100 before assay. Antibodies raised against the 148 kDa cartilage protein did not react with other cartilage macromolecules. Neither did they react with guanidinium chloride extracts of a number of non-cartilaginous tissues. The protein, then, is unique for cartilage. It was demonstrated in tracheal, nasal-septum, xiphisternal, auricular and epiphyseal cartilage. Surprisingly, the protein was detected neither in extracts of articular cartilage nor in extracts of the annulus fibrosus or the nucleus pulposus of the intervertebral disc.

The 148 kDa cartilage protein* was first identified in bovine tracheal cartilage, as a protein that co-fractionated with cartilage proteoglycans (Paulsson & Heinegård, 1979). The purified protein was found to contain three polypeptide chains, each having a molecular mass of 52 kDa (Paulsson & Heinegård, 1981). Its oligomeric structure requires intact disulphide bonds.

To understand the physiological function of the 148 kDa cartilage protein, a number of questions will have to be answered. Is it structurally related to any other cartilage macromolecule? Is it present only in cartilage? What is the specificity of its interaction with cartilage proteoglycans? In the study of these problems a method for the assay of the protein is required. An immunoassay method would provide both the necessary specificity and sensitivity. The design of such an assay method is complicated by the insolubility of the 148 kDa protein in solvents other than chaotropic salts and ionic detergents, e.g. sodium dodecyl sulphate. It has, however, been shown that proteins dissolved in sodium dodecyl sulphate may be detected by immunochemical methods, particularly when excess sodium dodecyl sulphate is bound in mixed micelles with a non-ionic detergent, such as Triton X-100 (Converse &

Papermaster, 1975). More recently, Caterson *et al.* (1979) developed a radioimmunoassay method, using a similar principle, in which cartilage link proteins were dissolved in a mixture of deoxycholate and Nonidet P-40.

Experimental

Purification of antigens

The 148 kDa protein was purified from bovine tracheal cartilage and its subunits were prepared by reduction and carboxymethylation as described elsewhere (Paulsson & Heinegård, 1981). Cartilage proteoglycan core protein, hyaluronic acid-binding region and link protein were prepared as described previously (Wieslander & Heinegård, 1979). Type II collagen was prepared from bovine nasal-septum cartilage by using a modification of the procedure of Miller (1972). The 36 kDa protein was isolated from bovine tracheal cartilage (M. Paulsson, Y. Sommarin & D. Heinegård, unpublished work) by using a side fraction obtained in the purification of the 148 kDa protein (Paulsson & Heinegård, 1981).

¹²⁵I-labelling of the 148 kDa cartilage protein

The 148 kDa protein was iodinated by a modification of the chloramine-T procedure of Greenwood *et al.* (1963). A stock solution (0.5 mg/ml) of the protein was made in 6 M-urea/5 mM-Tris/HCl buffer, pH 7.5. A 25 µl portion (containing 12.5 µg of protein) was mixed with 5 µl (0.5 mCi) of [¹²⁵I]iodide and 20 µl of chloramine-T (2 mg/ml) in

* The 148 kDa cartilage protein has previously (Paulsson & Heinegård, 1979, 1981) been referred to as the 'cartilage matrix protein'. An operational nomenclature is now introduced to allow simplified identification of novel non-collagenous cartilage proteins. Each such protein is designated by its molecular mass.

the urea/Tris/HCl buffer. The reaction was stopped after 60 s by addition of 100 μ l of sodium metabisulphite (1 mg/ml) in the urea/Tris/HCl buffer. The sample was diluted with 400 μ l of 1% (w/v) sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.5, and transferred to a column (V_i 10 ml) of Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). The column had been pretreated by elution with 2 ml of 1% (w/v) bovine serum albumin in the sodium dodecyl sulphate/phosphate buffer, and was subsequently eluted with the same buffer not containing bovine serum albumin. The iodinated 148 kDa protein was recovered from the void volume.

Preparation of antisera

Samples (150 μ g) of the 148 kDa protein, suspended in 0.9% NaCl, were used for immunization of rabbits, initially together with Freund's complete adjuvant and for booster after 1 month together with Freund's incomplete adjuvant. The animals were bled twice monthly, starting 1 month after the first booster. Sera were not purified further.

Second antibody was raised in sheep immunized with rabbit immunoglobulin G Fab fragments. The immunoglobulin fraction was isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Radioimmunoassay protocol

^{125}I -labelled 148 kDa protein, as well as samples to be analysed, were diluted with 0.1% bovine serum albumin/0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4. Samples of ^{125}I -labelled antigen solution (50 μ l containing 2–3 ng of the 148 kDa protein, i.e. 15 000–30 000 c.p.m.) and 50 μ l of test sample solution were mixed. Then 100 μ l of 4% (w/v) Triton X-100/10 mM-sodium phosphate buffer, pH 7.4, and 100 μ l of the appropriate dilution of the first antiserum in diluent buffer (0.5% bovine serum albumin/0.9% NaCl/0.1% NaN_3 /50 mM-sodium phosphate buffer, pH 7.5) were added. After mixing, the tubes were incubated for 20 h at 4°C. Subsequently 100 μ l of the appropriate dilution of the second antiserum in diluent buffer, containing 5% (w/v) poly(ethylene glycol) 6000, was added, and the tubes were incubated for another 20 h at 4°C. The mixture was diluted with 1 ml of the diluent buffer before centrifugation. The supernatant was decanted and the radioactivity of the precipitate was determined. Samples were assayed in triplicate and the average of the determinations was used for calculations.

Gel chromatography of a tracheal-cartilage extract

Bovine tracheal cartilage was extracted with guanidinium chloride as described previously (Paulsson & Heinegård, 1981). A sample of the extract was mixed with 9 vol. of ethanol and incubated

overnight at 4°C to precipitate proteins and proteoglycans. The precipitate was recovered by centrifugation in a bench-top centrifuge and washed twice with ethanol. It was redissolved in 1% sodium dodecyl sulphate/5 mM-sodium phosphate buffer, pH 7.4, and chromatographed on a column (0.8 cm $\times$ 138 cm) of Sepharose CL-4B (Pharmacia Fine Chemicals) eluted with the same buffer. Fractions (1.1 ml) were analysed for protein content with an automated version (Heinegård, 1973) of the Lowry procedure (Lowry *et al.*, 1951), with bovine serum albumin as standard. A sample of each fraction was diluted with 99 vol. of 0.1% bovine serum albumin/0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4, before quantitative determination of the 148 kDa protein by radioimmunoassay.

Preparation of tissue extracts

Samples of the following bovine tissues were obtained from 2-year-old animals directly after slaughter: cartilage from tracheal rings, nasal septum, xiphoid process of the sternum, ear, fetlock joint, knee joint (femoral surface) and intervertebral disc (anulus fibrosus as well as nucleus pulposus); aortic wall; skin; tendon; skeletal muscle; kidney and liver parenchyma; cornea, lens, sclera and vitreous body of the eye. Epiphyseal cartilage was from a calf about 6 months of age. The tissues were carefully dissected to prepare samples as pure and homogeneous as possible, and the pieces were frozen at –60°C until used. While still frozen, the pieces were finely minced with a scalpel.

The wet weight of each sample was recorded, and ice-cold 5 M-guanidinium chloride/5 mM-sodium phosphate buffer, pH 7.4, containing the proteinase inhibitors 5 mM-benzamidine chloride, 0.1 M-6-aminohexanoic acid, 10 mM-EDTA and 10 mM-N-ethylmaleimide, was added in a proportion of 20 ml/g of tissue. Extraction proceeded for 20 h at 4°C under gentle agitation. Unextracted material was removed by centrifugation at 9000 g_{av} for 30 min at 4°C. An extract of the mineralized matrix of adult bovine diaphyseal bone was prepared by Dr. A. Franzén (University of Lund). Ground bone had been pre-extracted, in a manner similar to that described above, to remove material not integral in the mineralized matrix. The matrix was then dissolved by extraction with a solvent as above, but also containing 0.25 M-EDTA (A. Franzén & D. Heinegård, unpublished work).

A 1 ml portion of each extract, corresponding to an equal amount of wet tissue, was mixed with 9 vol. of ethanol and kept at 4°C overnight. The precipitates were recovered by centrifugation in a bench-top centrifuge, briefly dissolved or suspended in 1 ml of 0.5 M-sodium acetate buffer, pH 7.0, and again precipitated with ethanol, as above, to remove

residual guanidinium chloride. These precipitates were dissolved in 5 ml of 0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4. Serial dilutions were made in 0.1% bovine serum albumin/0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4.

Dissociative CsCl-density-gradient centrifugation of cartilage extracts

Measured volumes (2.5–5.0 ml) of the cartilage extracts were adjusted to the density of 1.40 g/ml by addition of solid CsCl and diluted to 13.5 ml by addition of CsCl/extraction solvent of the same density. The samples were centrifuged at 38 000 rev./min (100 000 g, r_{av} , 6.1 cm) in the Beckman 70.1 Ti rotor for 60 h at 18°C. By using a tube slicer, the gradients were cut into a top fraction of 4.7 ml and a bottom fraction of 8.8 ml, corresponding to 35% and 65% respectively of the total volume. Measured samples of the top fractions, corresponding to an equal amount of wet tissue, were precipitated with ethanol, by using the same procedure as for whole extracts. The precipitates were analysed by radioimmunoassay and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Neville (1971), but with 1.5 mm-thick slab gels. The gels were 8.0% T $\times$ 2.5% C, where the first numeral (T) designates the total amount of monomer (% w/v) and the second numeral (C) designates the amount of *NN'*-methylenebisacrylamide, expressed as a fraction (% w/w) of total monomer in the gel casting solution. Identical samples were either treated with 2-mercaptoethanol, to reduce disulphide bonds, or not treated. Gels were stained for protein with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.). Apparent molecular weights were determined by comparison with reference proteins (high-molecular-weight and low-molecular-weight standard proteins: Pharmacia Fine Chemicals).

Results and discussion

Assay conditions

In initial experiments the different parameters of the assay method were tested. Radioimmunoassay in the presence of detergents was tested by using modifications of the assay for link proteins previously described (Wieslander & Heinegård, 1980). The presence of 0.2% sodium dodecyl sulphate/2% Triton X-100 in the antigen solution did not interfere with the assay. With this procedure we could omit the maleylation step, which was previously required to render the link proteins soluble. The assay may also be performed in dilute sodium dodecyl sulphate

without addition of Triton X-100. Preferably, however, Triton X-100 should be included to bind excess sodium dodecyl sulphate in mixed micelles and thereby decrease the risk of denaturation of immunoglobulins.

The sodium dodecyl sulphate/Triton X-100 solvent system worked equally well in the assay for the 148 kDa cartilage protein. The kinetics of the first and second antibody reactions were determined. In both cases maximal precipitation had been achieved at 20 h of incubation. The optimal ratio between first and second antibody was determined. Variations in temperature between 4°C and 24°C did not greatly affect the assay, and incubations were subsequently done at 4°C. With high concentrations of first

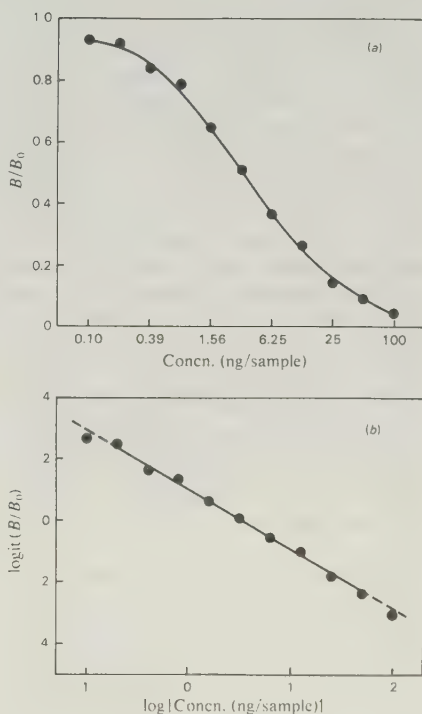


Fig. 1. Inhibition in the radioimmunoassay by 148 kDa cartilage protein standards

For full experimental details see the text. (a) Inhibition expressed as B/B_0 versus concentration in sample (logarithmic scale), where B is the radioactivity (c.p.m.) bound in sample determinations and B_0 is the radioactivity (c.p.m.) bound in the absence of competing antigen. (b) Inhibition expressed as $\text{logit}(B/B_0)$ versus $\log(\text{concentration})$ (Rodbard *et al.*, 1969).

antibody, 80–85% of the ^{125}I -labelled 148 kDa protein could be precipitated. A dilution of 1:3200 of the first antiserum gave approximately half-maximal precipitation and was used as a routine in inhibition experiments.

The inhibition curves were symmetrical (Fig. 1a), and half-maximal inhibition was obtained with about 3 ng/sample of competing antigen, a value in good agreement with the amount of iodinated antigen present. By using the logit/log plot procedure of Rodbard *et al.* (1969), a straight line was obtained (Fig. 1b). Such plots were subsequently used to calculate amounts. The assay could be used over a range from 0.2 to 50 ng/sample, even though the accuracy was expectedly higher in the middle portion of the range.

Effect of reduction and carboxymethylation of the 148 kDa cartilage protein

As the procedure for extraction and purification of the 148 kDa protein utilizes the denaturing conditions of 4 M- or 5 M-guanidinium chloride and the molecules are treated with 0.4% sodium dodecyl sulphate before assay, it is unlikely that the antigens are in a native state. The protein is, however, still recognized by the antibodies. It was therefore considered relevant to determine if the more extensively denatured subunits of the 148 kDa protein react with the antibodies raised against the intact protein. Such subunits were prepared by reduction and carboxymethylation (Paulsson & Heinegård, 1981). Compared with the intact protein, they were significantly less capable of inhibiting the binding of iodinated antigen (Fig. 2). The shape of the curve indicates that the subunits compete only for a fraction of the antibodies, and then with a lower affinity. This loss of antigenic determinants may be due to loss of tertiary and quaternary structure on dissociation of the trimeric complex and/or to interference of the carboxymethylated cysteine residues with antigenic sites.

Specificity of the assay method

To determine if the 148 kDa protein is antigenically related to other known cartilage macromolecules, inhibitory capacity in the assay was tested for proteoglycan core protein, proteoglycan hyaluronic acid-binding region, link protein, type II collagen and 36 kDa protein, a protein quantitatively prominent in tracheal cartilage (Paulsson & Heinegård, 1981). None of the preparations gave significant inhibition (results not shown), i.e. more than a 1000-fold excess compared with the amount of 148 kDa protein was required for inhibition. Inhibition at those high concentrations of competing antigen is probably due to minute (less than 0.1%) contaminations of 148 kDa protein antigen in the preparations used.

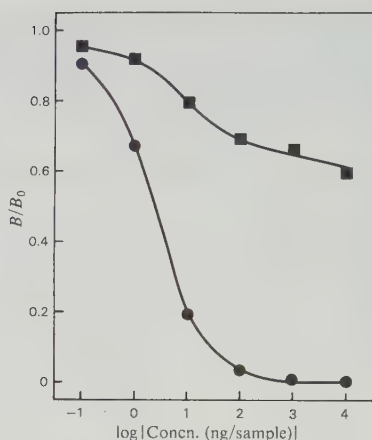


Fig. 2. Inhibition in the radioimmunoassay by the subunits of the 148 kDa protein (■) and the intact protein (●).

For full experimental details see the text. For definition of B/B_0 see Fig. 1 legend.

The specificity was further demonstrated by separating the proteins and proteoglycans obtained in a guanidinium chloride extract of bovine tracheal cartilage by gel chromatography on Sepharose CL-4B (Fig. 3). The 148 kDa protein was eluted as a distinct peak identified by the radioimmunoassay. The proteoglycans were eluted well separated at or close to the void volume, whereas the link proteins and the 36 kDa protein were eluted more retarded. Therefore the assay method appears to be highly specific for the 148 kDa protein, and this protein was found to be antigenically different from other matrix macromolecules.

Tissue distribution of the 148 kDa cartilage protein

Having demonstrated the specificity of the radioimmunoassay, it was then possible to determine the distribution of the 148 kDa protein among bovine tissues. 5 M-Guanidinium chloride, a solvent that efficiently extracts the 148 kDa protein from tracheal cartilage (Paulsson & Heinegård, 1981), was assumed to extract the protein also from other tissues. Proteins were recovered from the extracts by ethanol precipitation and dissolved in the 0.4% sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4, used in the assay. Serial dilutions made from extracts of the following non-cartilaginous tissues were tested in the radioimmunoassay (results not shown): bone, aortic wall, skin, tendon, cornea, lens, sclera, vitreous body, skeletal muscle, kidney and liver. None of these

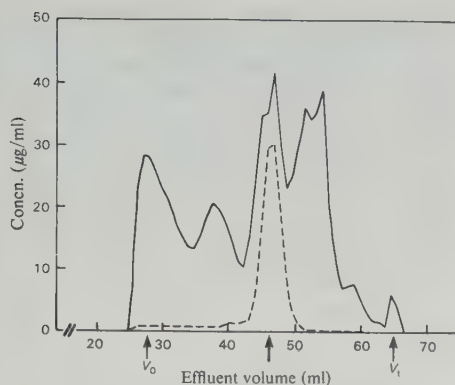


Fig. 3. Sepharose CL-4B gel chromatography of cartilage proteins in 1% sodium dodecyl sulphate/5 mM-sodium phosphate buffer, pH 7.4

For the preparation of the samples and other experimental details see the text. The curves designate total protein (—) and the 148 kDa cartilage protein, as determined by radioimmunoassay (---). The arrow indicates the elution position of purified 148 kDa protein, when chromatographed on the same column. V_0 and V_t indicate the void volume and the total volume respectively.

tissues contained detectable amounts of the 148 kDa protein, by using a procedure with a detection limit of about 1 µg of protein/g wet wt. of tissue. It appears that the 148 kDa protein is unique to cartilage.

Moderate inhibitions were obtained when dilution series of bovine sera were tested, corresponding to about 110 ng and 80 ng of the 148 kDa cartilage protein/ml in foetal and adult serum respectively (results not shown). It is possible that small amounts of the protein are released to the blood. These concentrations, however, are close to the detection limit in the assay. Therefore rather concentrated serum samples were used, and the inhibition could be due to unspecific interference by plasma proteins. In support, no distinct peak of inhibitory activity could be seen when serum was fractionated by ion-exchange chromatography (results not shown).

The cartilages analysed for content of the 148 kDa protein were from tracheal rings, nasal septum, xiphisternal process, articular surfaces of the fetlock and knee joints, epiphyseal growth plate, ear and the anulus fibrosus and nucleus pulposus of the intervertebral disc. Radioimmunoassay was done on material recovered by ethanol precipitation of 5 M-guanidinium chloride extracts, as described above for the non-cartilaginous tissues, but also on partially purified protein fractions obtained by direct dissociative CsCl-density-gradient centrifugation.

The density-gradient centrifugation was performed to remove proteoglycans and thereby allow parallel comparative analysis by radioimmunoassay and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Under the conditions used, all proteins are recovered in the top third of the gradient (Paulsson & Heinegård, 1981), and the radioimmunoassays of

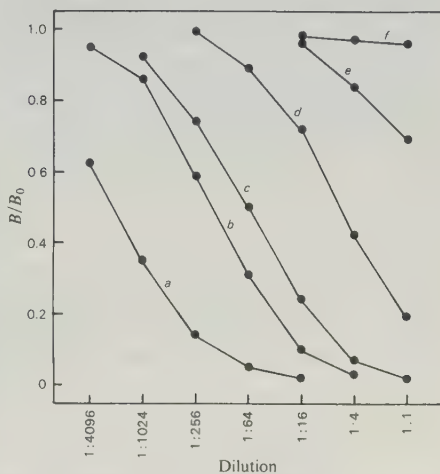


Fig. 4. Inhibition in the radioimmunoassay by samples from CsCl-density-gradient top fractions prepared from different bovine cartilages

For the preparation of the samples and other experimental details see the text. Samples were from tracheal (a), nasal-septum (b), xiphisternal (c), auricular (d), epiphyseal (e) and articular (f) cartilage. Each sample represents an equal wet weight of tissue. For definition of B/B_0 see Fig. 1 legend.

Table 1. Concentration of the 148 kDa protein in bovine cartilages determined with radioimmunoassay

Cartilage	148 kDa cartilage protein (mg/g wet wt. of tissue)
Tracheal	13.62
Nasal-septum	1.12
Xiphisternal	0.432
Auricular	0.036
Epiphyseal	0.003
Articular (fetlock joint)	Not detected
Articular (knee joint)	Not detected
Anulus fibrosus	Not detected
Nucleus pulposus	Not detected

the extracts and of the CsCl-density-gradient top fractions were accordingly in complete agreement. Some of the inhibition curves are depicted in Fig. 4. The amount of the 148 kDa protein in the extracts varies remarkably between the different cartilages. The protein is most enriched in tracheal cartilage (Table 1), but is present in comparatively large amounts also in nasal-septum and xiphisternal cartilages. Cartilage from ear and epiphyseal growth plate contain small amounts of the protein, and it could not be detected at all in articular cartilage or in any part of the intervertebral disc (Table 1).

The differential distribution of the 148 kDa protein among cartilages was confirmed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of some of the CsCl-density-gradient top fractions (Figs. 5a and 5b). The reduced subunits of the 148 kDa protein migrate with an apparent molecular weight of 60000 (Fig. 5a; Paulsson & Heinegård, 1981). A prominent band with this mobility was seen in the sample from tracheal cartilage and, gradually fainter, in the samples from nasal-septum and xiphisternal cartilages. Some of the other samples also gave bands of similar

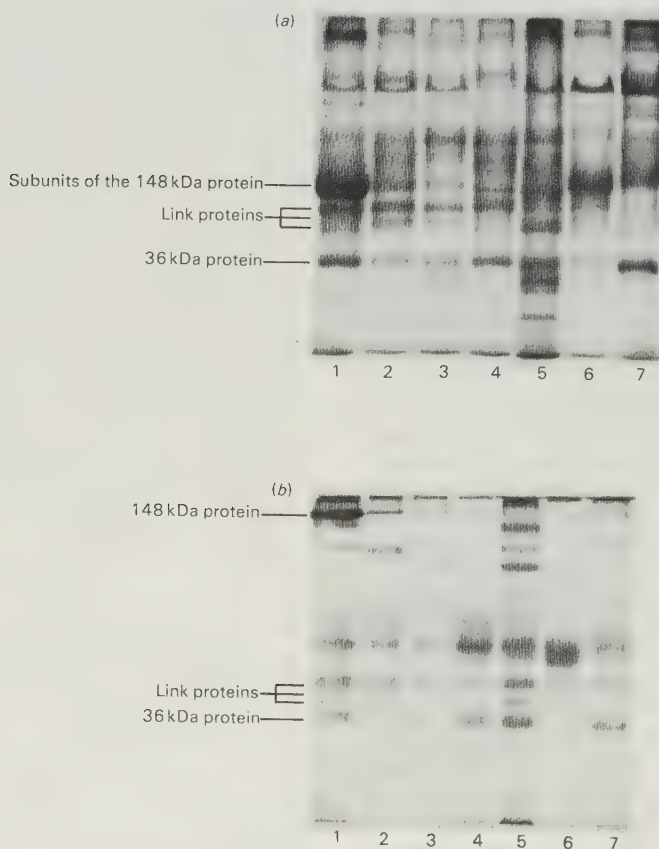


Fig. 5. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of samples from CsCl-density-gradient top fractions prepared from different bovine cartilages

For the preparation of the samples and other experimental details see the text. (a) Samples reduced with 2-mercaptoethanol. (b) Samples not reduced. Lane 1, tracheal cartilage; lane 2, nasal-septum cartilage; lane 3, xiphisternal cartilage; lane 4, auricular cartilage; lane 5, epiphyseal cartilage; lane 6, articular cartilage; lane 7, anulus fibrosus. Each sample represents an equal wet weight of tissue.

mobility. As the 148 kDa protein is an oligomeric protein, sensitive to reduction, further information could be gained by electrophoresis without prior reduction of samples (Fig. 5b). Under those conditions the 148 kDa protein is seen as a band close to the top of the gel. Again, this band was prominent in the sample from tracheal cartilage, and was present, but fainter, in the nasal-septum and xiphisternal cartilage samples. No material of similar mobility was seen in the other preparations. Samples from articular cartilage and intervertebral disc each contained a unique component of somewhat lower mobility than that of the link proteins. Whereas the content of the 148 kDa protein varies markedly between cartilages, the contents of link proteins and the 36 kDa protein appear to be relatively constant (Fig. 5a), indicating that the variation seen is not due to different extractability. It appears that the variation in 148 kDa protein content among the extracts represents a true difference in tissue content of the protein.

General discussion

According to morphological and gross biochemical analysis, different cartilages share many characteristics. The major molecular constituents, collagen and proteoglycans, are fairly constant from one cartilage to another, even though minor compositional differences are recognized. The formation of aggregates between proteoglycans, hyaluronic acid and link proteins also appears to be a constant feature of cartilage.

It was therefore surprising to find such marked differences between cartilages with regard to the amount of the 148 kDa protein present. The protein is not associated specifically with hyaline, fibrous or elastic cartilages. It is indeed difficult to relate the distribution pattern to other known parameters of cartilage composition and structure. This result, and

the observation of other proteins that appear prominent only in some of the cartilages (Figs. 5a and 5b), raises the question if there is a differentiation among cartilages expressed through their non-collagenous proteins. Some of these proteins, such as the link proteins and the 36 kDa protein (Fig. 5a), would then be common to all cartilages, whereas other proteins, such as the 148 kDa protein, would be subject to variation. Proteins of the second group could have functions specific for that very tissue and might be markers of cartilage differentiation.

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VARIATION IN QUANTITY AND EXTRACTABILITY OF THE 148-KILODALTON
CARTILAGE PROTEIN WITH AGE

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Summary

The occurrence of non-collagenous matrix proteins was studied in samples of tracheal cartilage from steers of different ages. The amounts of the 148 kDa cartilage protein were determined by radioimmunoassay of 4M guanidinium chloride extracts and of subsequent trypsin digests of the extraction residues. Surprisingly, the 148 kDa protein antigenicity was not changed by trypsin digestion, even though the protein was extensively degraded.

The amount of the 148 kDa protein increased dramatically with age, both in the guanidinium chloride extract and in the trypsin digest and reached maximal levels at about 3 and 8 years, respectively. The increase of the guanidinium chloride-soluble pool preceded that of the trypsin-soluble pool, possibly indicating a metabolic relationship. The ratio between the trypsin-soluble and guanidinium chloride-soluble pools increased continuously and at 12 years of age close to 90% of the total 148 kDa protein detected was insoluble in guanidinium chloride. At all ages, the bulk of the cartilage collagen was insoluble both to guanidinium chloride extraction and to trypsin digestion. The decreasing extractability of the 148 kDa protein was therefore not secondary to changes in the solubility of the collagen network.

Other cartilage proteins, such as the link proteins and the 36 kDa protein, showed much smaller quantitative variations partly of different character.

Introduction

Cartilage fulfills a number of important functions, being a part of the skeletal system and essential for its growth and development. Therefore much interest has been centered around the structure and composition of its major extracellular matrix components, collagen and proteoglycans (for refs. see Eyre, 1980; Heinegård & Paulsson, 1983). In recent studies, characterization has been extended to some predominant non-collagenous proteins present in cartilage.

One of these, the 148 kDa protein, is a glycoprotein composed of three similarly sized subunits, joined by disulphide bonds (Paulsson & Heinegård, 1981). Another, the 36 kDa protein, is also of glycoprotein nature and consists of a single polypeptide, rich in leucine (M. Paulsson, Y. Sommarin & D. Heinegård, unpublished results). While the 36 kDa protein appears to be uniformly present in most or all bovine cartilages, the 148 kDa protein is very prominent in some, i.e. tracheal and nasal cartilage, but can neither be detected in guanidinium chloride extracts of articular cartilage nor of the intervertebral disc (Paulsson & Heinegård, 1982). Both these proteins are synthesized by the chondrocytes (Paulsson et al., 1983). The 148 kDa protein appears to interact with proteoglycans in the matrix, indicated by the cofractionation with these molecules when isolated by non-denaturing procedures (Paulsson & Heinegård, 1979).

In a number of studies it has been shown that the composition and concentration of cartilage collagen and proteoglycans changes with the aging of the animal (for refs. see Inerot & Heinegård, 1982). Recently it was demonstrated that the variation in proteoglycan structure is largely due to gradual changes in the proportions between two distinct populations of aggregating proteoglycans (Inerot & Heinegård, 1983). Such changes could have a

definite impact on the physical properties of cartilage and do therefore provide a possibility of understanding the maturation and aging of the tissue in molecular terms.

In the present work we extend those observations by characterizing the changes occurring among prominent non-collagenous proteins in tracheal cartilage during the aging process.

Experimental

Extraction and fractionation of cartilage proteins:

The samples of bovine tracheal cartilage were those described previously (Inerot & Heinegård, 1983). They were from two male fetuses, 9 months of gestational age, and from steers 11, 22, 41, 75, 78, 96, 126 and 141 months of age. Tracheal rings had been dissected free from soft tissues and perichondrium shortly after slaughter, frozen in liquid N₂ and ground in a Wiley mill.

Samples of frozen cartilage (200 mg) were extracted for 24 hours at 30°C in 4 ml of 4M-guanidinium chloride/50 mM sodium acetate buffer, pH 5.8, containing the protease inhibitors 10 mM-EDTA, 0.1M-6-aminohexanoic acid, 5mM-benzamidinium chloride and 10mM-N-ethylmaleimide. The extraction residues were pelleted by centrifugation for 15 minutes at 3000 g_{av}, reextracted in the same buffer and then washed by repeated suspension in water.

The residues were suspended in 0.2M-sodium phosphate buffer, pH 8.0, containing 0.5 mg/ml of trypsin (TPCK-treated, from bovine pancreas, Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubated at 37°C for 48 hours under gentle agitation. Trypsin activity was then inhibited by the addition of phenylmethanesulphonyl fluoride to a final concentration of 10mM. After a brief incubation at room temperature, the trypsin-insoluble residue was

pelleted by centrifugation, as described above. The residue was extracted with guanidinium chloride, as above, washed with water and finally dissolved by incubation overnight at 60°C in 2.5 ml of 1M-NaOH. The guanidinium chloride extract of the trypsin-digested residue contained only minute amounts of protein and was therefore discarded.

In a separate experiment other portions (250 mg) of the same cartilage samples were extracted with 5 ml of the guanidinium chloride extraction buffer, as described above, and then briefly resuspended in 2.5 ml of the extraction buffer. The residues were dissolved in 1M-NaOH for determination of hydroxyproline contents, while the pooled supernatants were fractionated by direct caesium chloride density gradient centrifugation. Solid caesium chloride and additional extraction solvent was added to give a density of 1.36 g/ml in a final volume of 13.5 ml. The samples were centrifuged in a Beckman 70.1 Ti angle rotor at 40 000 rpm (r_{av} 6.1 cm, 109 000 g) for 60 hours at 18°C. The top third of the gradient was separated using a Beckman tube slicer. Under the conditions used this fraction will contain all cartilage proteins and virtually no proteoglycans (Heinegård et al., 1981).

Radioimmunoassay:

The amounts of 148 kDa protein were determined by use of the radioimmunoassay described previously (Paulsson & Heinegård, 1982). Briefly, the procedure involves iodination of the protein by the chloramine T method (Greenwood et al., 1963) and dissolving the ^{125}I -antigen and the samples and standards to be assayed in an 0.1%-bovine serum albumin/0.4%-sodium dodecyl sulphate/10 mM-sodium phosphate buffer, pH 7.4. Excess sodium dodecyl sulphate is bound in mixed micelles by the addition of a tenfold excess of Triton X-100. The assay is then performed using a double antibody system. All samples were assayed in triplicates and at three different dilutions.

Before assay the samples had to be transferred to the sodium dodecyl sulphate-solvent. This was accomplished using precipitation with ethanol as described elsewhere (Paulsson et al., 1983). The precipitates were dissolved and diluted in the sodium dodecyl sulphate-containing buffer used for radioimmunoassay. Samples from the trypsin-digests were made 0.1% with regard to ovomucoid, to further ensure complete inhibition of trypsin, and 0.4% with regard to sodium dodecyl sulphate, before dilution in the radioimmunoassay buffer.

The proteins extracted with guanidium chloride were analysed in the radioimmunoassay for the 148 kDa protein also after trypsin digestion. Samples of the extracts were precipitated with ethanol (Paulsson et al., 1983) and the precipitates were suspended in a volume of 0.2M-sodium phosphate buffer, pH 8.0, containing 0.5 mg/ml trypsin, identical to the original volume of the sample. Digestion and subsequent handling of the digests were the same as described for the residues.

Samples for the determination of link protein were pretreated and dissolved in an identical manner and assayed using the same procedure as in the 148 kDa protein assay, but with an antiserum specific for the link proteins (Wieslander & Heinegård, 1979, 1980).

Gel electrophoresis:

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis was performed as described by Neville (1971), but using 1.5 mm thick slab gels. The gels were 8.0% x 2.5%, where the first numeral (T) designates the total amount of monomer (% w/v) and the second numeral (C) designates the amount of NN'-methylenebisacrylamide, expressed as a fraction (% w/w) of total monomer in the gel casting solution. Identical samples were either treated with

2-mercaptoethanol for 2h at 37°C, to reduce disulphide bonds, or not treated prior to electrophoresis. Each sample represented an equal amount of tissue, corresponding to 25 µg of hydroxyproline, as determined in the extraction residues. Gels were stained for protein with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.). Apparent molecular weights were determined by comparison with reference proteins (high-molecular-weight and low-molecular weight standard proteins; Pharmacia Fine Chemicals, Uppsala, Sweden).

Hydroxyproline determination:

Residues were dissolved by incubation overnight at 60°C in 2.5 ml of 1M-NaOH. The samples were neutralized by the addition of 0.75 ml of 5M-HAc. Portions of the guanidinium chloride extracts were precipitated with ethanol (Paulsson et al., 1983) briefly dissolved in 1M-NaOH and neutralized with 5M-HAc, as with the residues. Samples from the trypsin-digests required no pretreatment. Hydrolysis was done in 6M-HCl at 100°C for 24 hours and the contents of hydroxyproline were determined by the procedure of Stegeman and Stalder (1967).

Results and discussion

Solubilization and quantitation of the 148 kDa protein:

In previous studies the 148 kDa protein has been solubilized from the tissue by extraction with 4 or 5M guanidinium chloride. Even though such solvents do extract considerable amounts of the protein from bovine tracheal and nasal cartilages (Paulsson & Heinegård, 1982), it has not been known if all of the 148 kDa protein present in the tissue is recovered. When planning the present study, aiming at identifying changes occurring among cartilage non-collagenous proteins with aging of the animal, we therefore wanted to determine if additional 148 kDa protein

remains in the residue after extraction with guanidinium chloride. We found that trypsin digestion of extraction residues releases large amounts of protein fragments that react in the radioimmunoassay for the 148 kDa protein. Surprisingly, such a digest, as well as a digest of the purified protein, completely inhibited the binding of ^{125}I -labelled 148 kDa protein, showing that all antigenic determinants recognized on the intact protein were still present, although no intact 148 kDa protein could be demonstrated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (results not shown). Control digestions containing only trypsin did not affect the assay, when treated with the same combination of proteinase inhibitors as the samples.

To determine if the quantitative response in the assay obtained with the tryptic peptides was similar to that of the intact protein, proteins present in guanidinium chloride extracts of tracheal cartilage were analysed with or without prior trypsin digestion of the samples. The conditions (1:100 relation between enzyme and the wet weight of the original cartilage sample, 48 hours of incubation at 37°C) were chosen to ensure limit digestion. The inhibitions obtained with and without trypsin treatment were almost identical and the sample dilutions gave slopes closely similar to that of the standard curve (Fig. 1). It was concluded that the tryptic peptides could be quantitated without any changes in the assay conditions and that the values obtained with digests were representative for the amount of intact 148 kDa protein originally present.

Variation in quantity of the 148 kDa protein with age:

The amounts of 148 kDa protein in 4M guanidinium chloride extracts and in trypsin digests of the extraction residues were determined for tracheal cartilage samples from steers ranging from 0 to 12 years of age (Fig. 2). The values given for the guanidinium chloride extracts were determined after trypsin

digestion of ethanol-precipitated aliquots to assure that the data on extracts and extraction residues would be altogether comparable, but samples of extracts were also assayed without digestion and gave values in good agreement.

Comparatively small amounts of 148 kDa protein were present in the samples from the youngest animals (Fig. 2a). The guanidinium chloride-soluble as well as the trypsin-soluble pools of the protein then increase markedly with age, to reach maxima at 3-4 years and 6-10 years, respectively. Both pools again decrease at high ages. The ratio between the guanidinium chloride-soluble and the trypsin-soluble pools of the 148 kDa protein increases continuously (Fig. 2b), the changes being most pronounced between 3 and 8 years of age.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of cartilage proteins:

The variation of the quantity of the 148 kDa protein in the guanidinium chloride extracts could be confirmed by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (Fig. 3). Prior to electrophoresis the protein fraction was partially purified by direct dissociative caesium chloride density gradient centrifugation in order to remove proteoglycans which otherwise cause the stacking gel to collapse. The 148 kDa protein is easily identified because of its trimeric structure, that causes a shift in mobility between samples treated (Fig. 3a) or not treated (Fig. 3b) with 2-mercaptoethanol. Some material remains at the position of the nonreduced 148 kDa protein also after reduction. As this band shows similar variation in intensity as the 148 kDa protein subunits it could bear some relation to this protein. Among other previously described proteins, the 36 kDa protein shows a somewhat increasing staining intensity during growth and maturation and then remains rather constant. Interestingly, its mobility changes slightly at early age.

Quantitation of link proteins:

The amount of link proteins is more difficult to assess from sodium dodecyl sulphate/polyacrylamide gel electrophoresis, because of their lesser abundance and their polymorphism. They were therefore separately quantified by radioimmunoassay (Fig. 4). A slight increase occurs with increasing age, but the changes are much less pronounced than those observed with the 148 kDa protein and more similar to those estimated for the 36 kDa protein. The increase in contents of link proteins is of similar magnitude as that of the glycosaminoglycan contents earlier described (Inerot & Heinegård, 1983), which indicates that the ratio between link proteins and proteoglycans remains relatively constant in the tissue.

Solubility of collagen:

An increasing stability of the collagen network could lead to entrapment of other molecules with ensuing, decreased extraction yields. Therefore, the distribution of collagen between the guanidinium chloride extract, the trypsin digest and the trypsin-insoluble residue was determined and plotted as a function of age (Fig. 5). The bulk of the collagen remains insoluble at all ages. The guanidinium chloride-soluble and trypsin-soluble pools constitute a somewhat larger fraction in early years, but the differences are minute when compared with the changes in quantity and solubility of the 148 kDa protein and do probably not have any bearing on these parameters.

General discussion

The apparently complete insensitivity of the antigenicity of the 148 kDa protein to trypsin digestion is remarkable, particularly as the protein is being extensively degraded (results not shown). One possible explanation would be that the oligosaccharides present on the protein are major antigenic determinants. The fact that the antigenicity is much decreased after reduction and alkylation (Paulsson & Heinegård, 1982) does, however, indicate that the determinants are peptide in nature and probably depend on higher order structure. The other possible explanation is that the antiserum is only directed against determinants present in one or several, comparatively small, trypsin-resistant regions of the protein. If so, these regions are probably stabilized by disulphide bonds.

Even though large amounts of peptides, reacting in the 148 kDa protein radioimmunoassay, could be released by trypsin digestion, the tissue could not be completely solubilized with less than incubation in 1M-NaOH at elevated temperature. It is therefore possible that a portion of the 148 kDa protein is still not accounted for and, for example, the decrease in 148 kDa protein seen at high age should therefore be interpreted with some caution.

All values, given in the present text, for amounts of non-collagenous proteins are related to the amount of collagen in the tissue sample, as determined by its content of hydroxyproline. It was not possible to obtain representative data on the wet weights, as the samples were collected over a rather long period of time and loss of water occurs during storage in the freezer. There was, however, a tendency for the samples from the older

animals to contain less collagen per weight unit. Therefore a slight increase in amount relative to hydroxyproline could mean a constant amount relative to wet weight.

The guanidinium chloride-soluble pool of 148 kDa protein reaches its maximum at considerably younger age than does the trypsin-soluble pool. An attractive interpretation of the presently available data is that the guanidinium chloride-soluble pool of 148 kDa protein is a transient one, containing relatively recently secreted molecules, some of which will eventually be laid down in the matrix in a manner that renders them insoluble to extraction with guanidinium chloride. The mechanism by which the 148 kDa protein becomes unextractable is not known, but it is possible that, in analogy with collagen, it may depend on the formation of covalent crosslinks.

Acknowledgements

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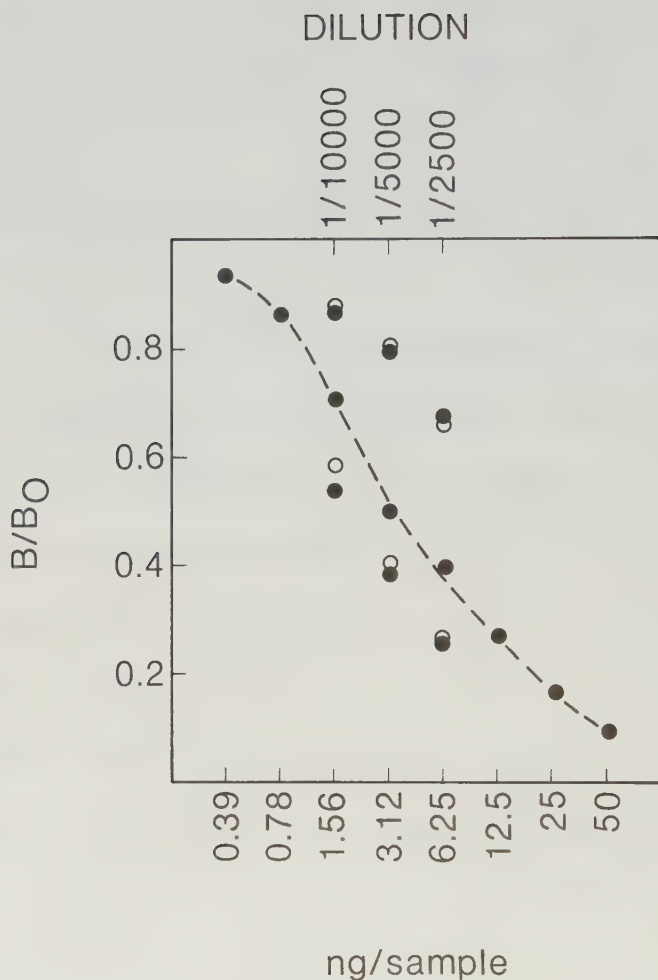


Fig. 1:

Inhibition in the 148 kDa protein radioimmunoassay by guanidinium chloride extracted proteins, digested (O) or not digested (●) with trypsin.

Dilutions of samples from two extracts, containing high and low quantities of 148 kDa protein, respectively, are compared with a dilution curve of purified, intact 148 kDa protein (●-●-). Inhibition is expressed as B/B_0 , where B is the radioactivity (c.p.m.) bound in sample determinations and B_0 is the radioactivity bound in the absence of competing antigen. For full experimental details see the text.

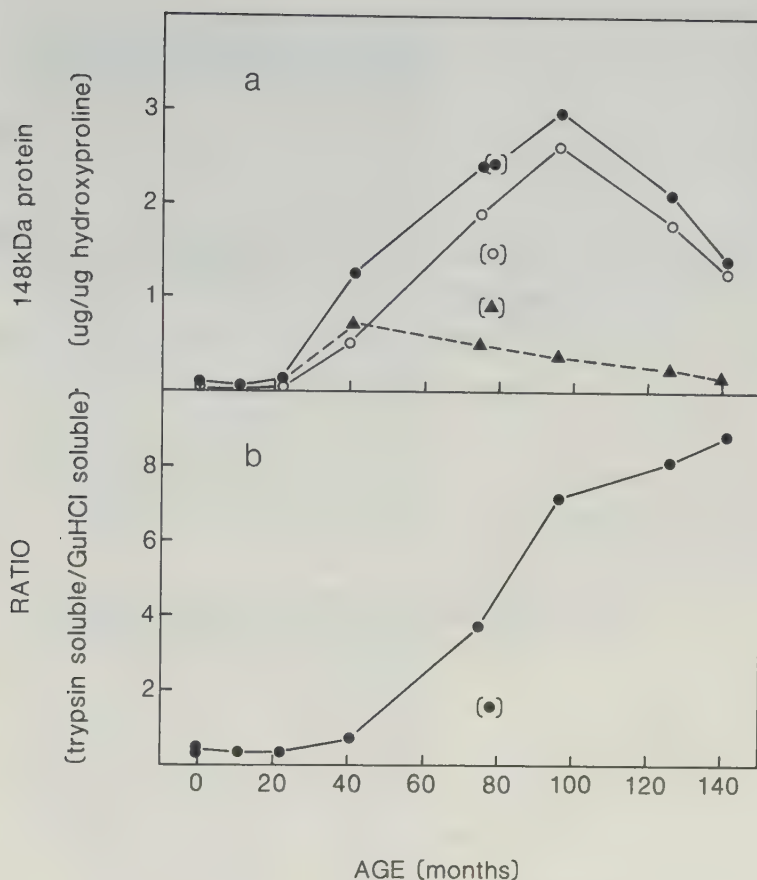


Fig. 2:

Variation in contents of the 148 kDa protein in bovine tracheal cartilage with age.

a. The amount of the 148 kDa protein detected in guanidinium chloride extracts (—▲—), in trypsin-digests of the extraction residues (—○—) and the total detectable amount of the protein (—●—) was determined by radioimmunoassay of tryptic peptides and related to the amount of hydroxyproline present in the tissue samples.

b. The ratio between the trypsin-soluble and guanidinium chloride-soluble pools of the 148 kDa protein was calculated from the data given in a.

One sample (78 months of age) consistently gave an aberrant partition between guanidinium chloride-soluble and insoluble protein. The data points for this sample are given within brackets. For full experimental details see the text.

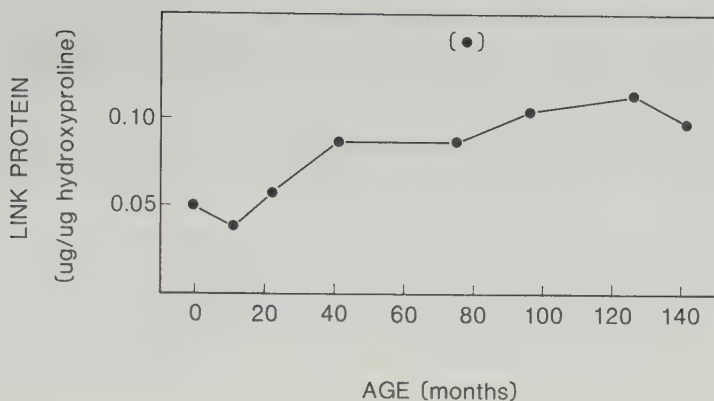


Fig. 4:

Variation in contents of link protein in guanidinium chloride extracts of bovine tracheal cartilage with age.

The amounts of link protein were determined by radioimmunoassay and related to the amount of hydroxyproline in the tissue samples. For full experimental details see the text.

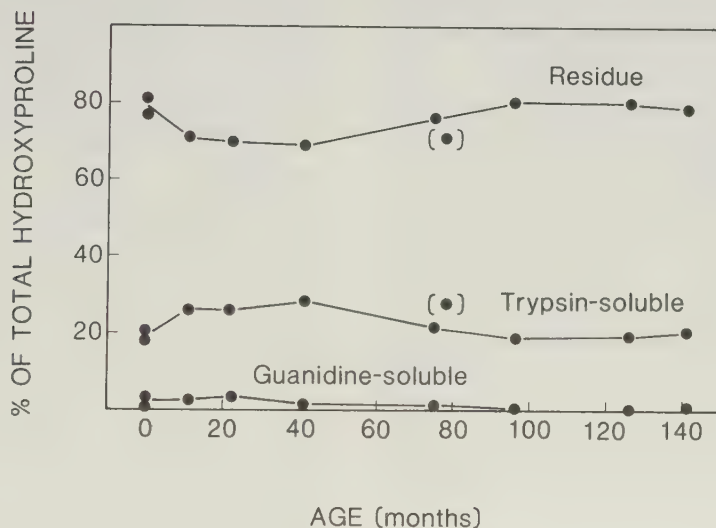


Fig. 5:

Variation in the recovery of collagen in the guanidinium chloride extract, the subsequent trypsin digest and the insoluble residue with age.

Collagen in extracts and residues was determined as the amount of hydroxyproline released by acid hydrolysis. For full experimental details see the text.

Metabolism of cartilage proteins in cultured tissue sections

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The biosynthesis and turnover of cartilage proteins was studied in organ cultures of bovine tracheal-cartilage sections. In cultures labelled with [^3H]leucine, more than 99% of the labelled macromolecules were retained in the sections. About half of the [^3H]leucine-labelled protein was extracted with 4 M-guanidinium chloride. The incorporation of [^3H]leucine into protein extractable with guanidinium chloride was linear with time, after an initial delay of 20–25 min. The 148 kDa and 36 kDa cartilage proteins were major labelled components in this extract. The elimination of the proteins was studied by using a pulse–chase protocol. The 148 kDa protein was found to have a very slow turnover, similar to that of proteoglycans, whereas the 36 kDa protein was eliminated more rapidly.

The cartilage extracellular matrix contains collagen, proteoglycans and a limited number of non-collagenous proteins (Paulsson & Heinegård, 1979, 1982). Among the non-collagenous proteins, the link proteins have a well-defined function in stabilizing the interaction between proteoglycan monomers and hyaluronic acid, important for the formation of proteoglycan aggregates (Heinegård & Hascall, 1974; Hardingham, 1979; Franzén *et al.*, 1981).

In recent years two additional non-collagenous proteins have been identified in cartilage extracts. One of these, the 148 kDa protein [this protein has in two previous papers (Paulsson & Heinegård, 1979, 1981) been referred to as the 'cartilage matrix protein'], is a trimer of similarly sized disulphide-bonded subunits (Paulsson & Heinegård, 1981). It is quantitatively prominent in guanidinium chloride extracts of bovine tracheal and nasal cartilages, but it is not detected in extracts of either articular cartilage or intervertebral disc (Paulsson & Heinegård, 1982). Available data indicate that the 148 kDa protein interacts with proteoglycans (Paulsson & Heinegård, 1979), although the specificity of the interaction has not been demonstrated. It is likely that the 148 kDa protein is a structural component of the extracellular matrix, particularly in tracheal cartilage, where it constitutes as much as 1% of the tissue wet weight.

Another non-collagenous cartilage protein, the 36 kDa protein, consists of a single polypeptide chain with a high content of leucine (about 140 residues/1000 residues; M. Paulsson, Y. Sommarin & D. Heinegård, unpublished work). It appears to be

present in all bovine cartilages (Paulsson & Heinegård, 1982).

Extracellular components of cartilage are synthesized by the chondrocyte or may enter the cartilage by diffusion from surrounding tissues or the blood, even though most cartilages contain few blood vessels. Examples of structural components synthesized by the chondrocyte are proteoglycans and collagen (for references see Eyre, 1980; Heinegård & Paulsson, 1983). These molecules have a very slow turnover once they are integrated in the cartilage matrix. In different studies of their turnover, half-lives ranging from 1 week to months and perhaps even years have been determined (Mankin & Lipiello, 1969; Maroudas, 1975, 1979; Lohmander, 1977). Other cartilage macromolecules, if structural components of the tissue matrix, would by analogy be expected to be synthesized by the chondrocyte and to show a slow turnover in the tissue. The present work was initiated to study the synthesis and turnover of non-collagenous cartilage proteins, particularly the 148 kDa and the 36 kDa proteins, and to relate the findings to simultaneously obtained data on proteoglycan core protein and link proteins.

Experimental

Materials

Ham's F12 H medium (GIBCO, Grand Island, NY, U.S.A.) was buffered with 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (Sigma Chemical Co., St. Louis, MO, U.S.A.). The unlabelled leucine normally included in the medium

was omitted, to allow a high specific radioactivity of [^3H]leucine.

Ham's F12 HA medium, used in chase experiments, was buffered with 10mM-Hepes and supplemented with '50 $\times$ essential amino acids for Eagle's minimum essential medium' (20 ml/l) (Flow Laboratories, Rockville Pike, MD, U.S.A.) and 2mM-leucine. The medium was further supplemented with penicillin (50 i.u./ml) and streptomycin (50 μg /ml) (both from Flow Laboratories).

[^3H]Leucine (65 or 160 Ci/mmol) and carrier-free [^{35}S]sulphate were from The Radiochemical Centre, Amersham, Bucks., U.K.

Cartilage sections were cultured in 50 ml Falcon conical polypropylene tubes (Becton Dickinson Labware, Oxnard, CA, U.S.A.) or in Linbro tissue-culture multi-well plates (Flow Laboratories) with 16 mm-diameter wells.

Organ cultures

Bovine tracheas from animals about 2 years old were obtained within 1 h after slaughter. Exposed surfaces were briefly washed with 70% (v/v) ethanol, and the tracheal rings were dissected free from mucosa and perichondrium under aseptic conditions. Transverse sections, 1 mm thick, were cut from the central portions of the rings, by using two fixed parallel blades. The sections thus obtained were of reproducible size and had an average wet weight of about 50 mg.

Four types of protocols were used for culture.

Pulse experiments. Sections were incubated in a 50 ml tube in 1 ml of Ham's F12 H medium (containing 20 μCi of [^3H]leucine and 10 μCi of [^{35}S]sulphate/ml)/section. To allow equilibration with radioisotope, the cultures were preincubated under gentle agitation at 3°C for 60–90 min before starting the pulse. The temperature was then rapidly raised by heating the tube for 15 min at 37°C in a water bath, whereafter the pulse was continued in a 37°C incubator. Sections were removed and immediately frozen at the times indicated.

In a subset of the pulse experiments, diffusion of radioisotopes into the cartilage as well as the time course of their early incorporation into macromolecules was studied. In this case the sections were preincubated in Ham's F12 H medium for 3 h at 37°C. At this time [^3H]leucine (20 μCi /ml) and [^{35}S]sulphate (10 μCi /ml) were added to the culture. Sections were then removed at the times indicated and immediately frozen.

Pulse-chase experiments. Sections were incubated in a 50 ml tube in medium containing radioisotopes as discussed above for pulse experiments. After 6 h the medium was replaced by Ham's F12 HA (2 ml/section) supplemented with leucine (2 mM) but not containing radioisotopes. Medium

was changed every 4 days. Sections were removed at the times indicated and immediately frozen.

In a separate experiment release of labelled macromolecules to the medium was studied. Both pulse and chase were done with one section in each well of a multi-well plate. Labelling was done with 1 ml of medium/section and chase with 2.5 ml/section, by the protocol described above for pulse-chase.

Isolation of cartilage proteins

Radiolabelled cartilage sections were cut into pieces with no side exceeding 2 mm. In one experiment the sections were instead cut into 0.2 mm-thick slices, with a Mickle gel slicer. Both procedures gave the same extraction yield. Each section was extracted under continuous stirring at 3°C for 24 h with 4 ml of 4M-guanidinium chloride/50mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors 5mM-benzamidinium chloride, 0.1M-6-aminohexanoic acid, 10mM-EDTA and 4mM-N-ethylmaleimide. The extraction residue was pelleted by centrifugation for 15 min at 3000 g_{av} , and the extract was carefully removed. In some experiments the residue was extracted for two more 24 h periods, each time with 2 ml of the guanidinium chloride extraction buffer. Subsequently, no more labelled material could be released by guanidinium chloride extraction. The final residues were dissolved by incubation overnight at 60°C in 1 ml of 1M-NaOH. The samples were neutralized by the addition of 0.3 ml of 5M-acetic acid and adjusted to a final weight of 5 g by the addition of water. Duplicate samples were hydrolysed in 6M-HCl at 100°C for 24 h, and the content of hydroxyproline was determined by the procedure of Stegeman & Stalder (1967). Other samples were taken for liquid-scintillation counting to determine the amount of radioactivity incorporated into unextractable protein. A ratio of scintillant (Instagel; Packard Instruments, Downers Grove, IL, U.S.A.) to sample of 10:1 was used for all determinations of radioactivity.

Extracts of cartilage sections were fractionated by density-gradient centrifugation in CsCl/4M-guanidinium chloride. Solid CsCl and extra extraction solvent were added to give a density of 1.36 g/ml in a final volume of 13.5 ml. The samples were centrifuged in a Beckman 70.1 Ti angle rotor at 38 000 rev./min (100 000 g_{av} , r_{av} 6.1 cm) for 60 h at 17°C. The tubes were divided in four fractions of equal volume with a Beckman tube-slicer.

The amount of radioactive macromolecules in extracts and CsCl-density-gradient fractions was determined by ethanol precipitation. Samples (50 μl) were diluted with 150 μl of water and precipitated with 1800 μl of 95% (v/v) ethanol. The precipitates were washed with additional ethanol and dissolved in

1 ml of 4 M-guanidinium chloride. Scintillant was added and the radioactivity determined. Free radioisotope in the sections was quantified by concomitant determination of the radioactivity remaining in the supernatant from the ethanol precipitation step. The radioactivity was always related to the amount of hydroxyproline present in the extraction residue.

Gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Neville (1971) on 3 mm-thick slab gels. The gels were 8.0% $\times$ 2.5%, where the first numeral (T) designates the total amount of monomer (% w/v) and the second numeral (C) designates the amount of *NN'*-methylenebisacrylamide, expressed as a fraction (% w/w) of total monomer in the gel casting solution. Samples of the pooled two top fractions from the CsCl density gradients, corresponding to 1 mg wet wt. of tissue, were precipitated with ethanol, as described above, redissolved in 0.5 M-sodium acetate buffer, pH 7.0, and again precipitated with ethanol to remove the last traces of CsCl and guanidinium chloride. The precipitates were then dissolved in 50 μ l of sample 'cocktail' (Neville, 1971) and incubated at 37°C for 2 h before electrophoresis. Samples to be reduced contained 5% 2-mercaptoethanol. The gels were stained for protein with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.). The positions of protein standards and proteins endogenous to samples were recorded with a Zeineh soft-laser densitometer. The lanes containing radioactive samples were then excised and sliced in 2 mm sections with a Mickle gel-slicer. Slices were dissolved by treatment with 500 μ l of 30% (w/v) H_2O_2 , either at 80°C for 2 h or at 37°C overnight. Scintillant was added and the radioactivity determined in a liquid-scintillation counter.

Results and discussion

Retention of the [3H]leucine-labelled macromolecules in the tissue

More than 99% of the incorporated [3H]leucine was retained in the cartilage when sections were labelled for up to 6 h. When identical sections were prelabelled for 6 h and then 'chased' for 190 h, only 3% of the total macromolecular radioactivity appeared in the medium. The [3H]leucine-labelled proteins were retained in the matrix of the sections in a manner similar to the situation *in vivo*, of importance for studies of the metabolism of these proteins. Because of the small proportion of the radioactive material released from the sections, media were not analysed in subsequent experiments.

Extraction of [3H]leucine-labelled macromolecules

About 50% of the [3H]leucine incorporated into the cartilage sections during labelling for up to 6 h was extracted with 4 M-guanidinium chloride. When sections prelabelled for 6 h were 'chased' for up to 188 h, the proportion of extractable radioactivity decreased to about 40%, even though the absolute amount of radioactivity decreased also in the residue. In separate experiments it was shown that the yield of 148 kDa protein was not higher when the guanidinium chloride concentration was increased to 5 or 6 M. The extraction yield of 3H -labelled proteins could be increased by about 25% of the total 3H label in the continuously labelled sections and by about 15% in the pulse-chase-labelled cartilage by repeated extraction with 4 M-guanidinium chloride (see the Experimental section). Most of the radioactive material recovered by a second extraction was high-molecular-weight polypeptides, distinct from the 148 kDa and 36 kDa proteins (results not shown), as shown by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The amount of [3H]leucine label in the 148 kDa and 36 kDa proteins was low compared with that in the first extract and appeared to be of a constant proportion, probably representing a small amount of first extract trapped in the extraction residue. Only the first guanidinium chloride extracts were analysed in subsequent experiments.

Kinetics of [3H]leucine and [^{35}S]sulphate incorporation

Cartilage sections were incubated with [3H]leucine and [^{35}S]sulphate and extracted with 4 M-guanidinium chloride. Macromolecules were separated from unincorporated radioisotope by ethanol precipitation, and the amounts of incorporated and of free radioisotope were determined. Labelled macromolecules appeared in the guanidinium chloride-extractable pool after less than 10 min, with both [3H]leucine (Fig. 1a) and [^{35}S]sulphate (Fig. 1b). The increase in both 3H - and ^{35}S -labelled macromolecules became linear with time after about 40 min and remained so for the duration of the experiment. When the linear portions of the curves were extrapolated to zero radioactivity, lag times of 20–25 min were obtained for both radioisotopes (Fig. 1). Determination of the radioactivity in the supernatants from the ethanol precipitation step indicated rapid equilibration of both radioisotopes between medium and cartilage sections, i.e. the amount of free radioisotope in the sections had already reached 70% of the plateau value after 5 min (Fig. 1).

The lag phase must therefore at least partly be due to factors other than isotope diffusion. In similar experiments, with slices of pig laryngeal cartilage, Hardingham & Muir (1972) demonstrated a delay of

40 min before ^{35}S -labelled proteoglycans became extractable. From this observation and radioautography results, they concluded that proteoglycans did not become extractable with guanidinium chloride until after secretion from the cells. The lag phase of 22 min that we observed for the extractable

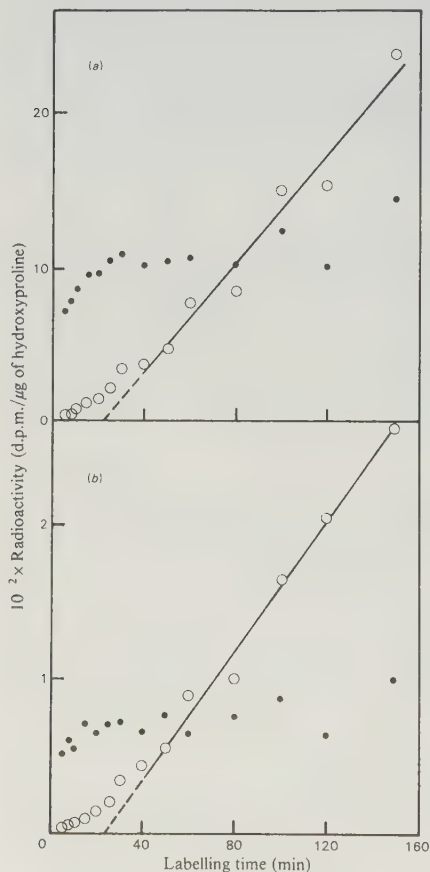


Fig. 1. Kinetics of incorporation of ^3H leucine (a) and ^{35}S sulphate (b) into guanidinium chloride-extractable macromolecules

Radiolabelled cartilage sections were extracted with 4M-guanidinium chloride/50mM-sodium acetate buffer, pH 7.4, and the radioactivity in macromolecules (○) as well as free radioisotope (●) was determined after ethanol precipitation. The delay in appearance of labelled macromolecules in the extractable fraction was determined by extrapolation of the linear portion of the incorporation curves.

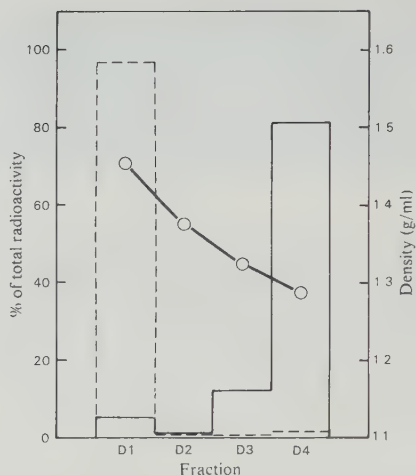


Fig. 2. Fractionation of ^3H leucine- and ^{35}S sulphate-labelled macromolecules by CsCl-density-gradient centrifugation in 4M-guanidinium chloride

The example given is of sections labelled for 6 h, before extraction with 4M-guanidinium chloride/50mM-sodium acetate buffer, pH 5.8. The separations obtained at other labelling times and in chase experiments were closely similar. —, ^3H Leucine; ---, ^{35}S sulphate; ○—○, density (g/ml).

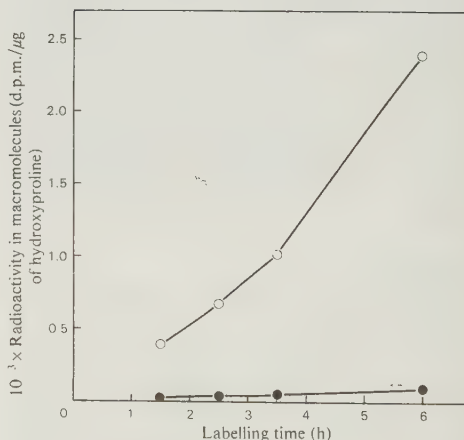


Fig. 3. Accumulation of ^3H leucine-labelled macromolecules in the tissue

Synthesis of proteoglycans (●) and proteins (○) was determined as the ^3H radioactivity in the D1 fraction and the pooled D3 and D4 fractions respectively obtained by CsCl-density-gradient centrifugation of 4M-guanidinium chloride extracts (Fig. 2).

[^3H]leucine-labelled protein is similar to the time from introduction of radioisotope to export of labelled protein when determined for typical secretory proteins. For example, the lag phase for [^3H]leucine-labelled serum albumin has been deter-

mined to be 19–21 min in isolated hepatocytes and 22–26 min in perfused rat liver (Feldhoff *et al.*, 1977). It therefore appears that the labelled molecules in the guanidinium chloride extracts are predominantly extracellular in origin.

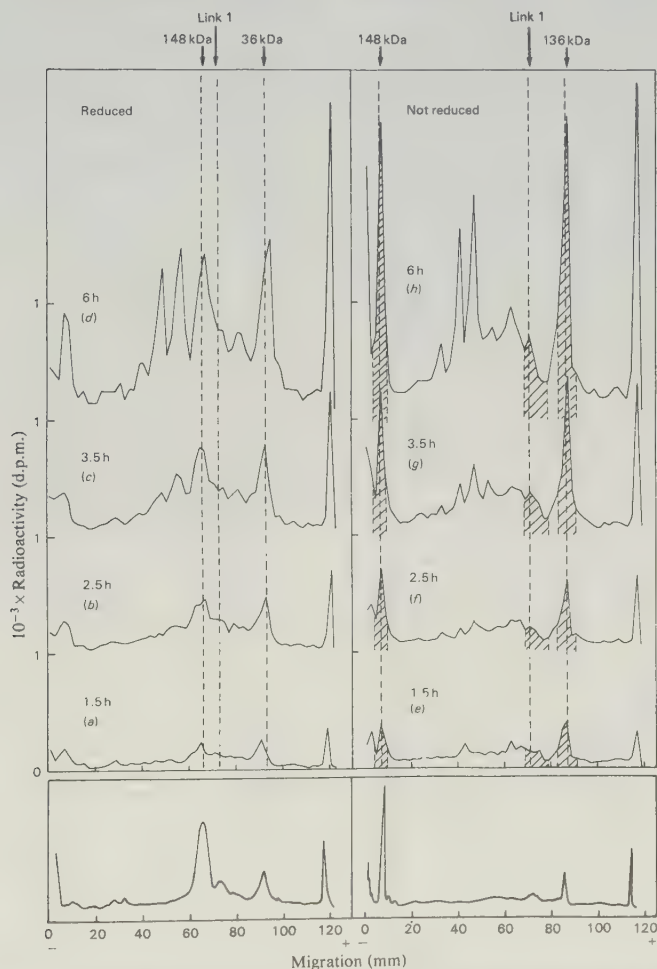


Fig. 4. Fractionation of [^3H]leucine-labelled cartilage proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Samples were obtained by ethanol precipitation of the pooled D3 and D4 fractions from CsCl -density-gradient centrifugation of 4 M-guanidinium chloride extracts. Cartilage sections had been labelled with radioisotope for the times indicated in the Figure. Electrophoresis was done on samples corresponding to 1 mg wet wt. of tissue, either reduced with 2-mercaptoethanol (a–d) or not reduced (e–h). Radioactivity profiles were obtained by liquid-scintillation counting of solubilized gel slices. The amounts of [^3H]leucine incorporated into individual proteins were calculated as indicated by the hatched areas. In the lower frame examples are given of the protein profiles obtained by Kenacid Blue staining of the samples reduced with 2-mercaptoethanol (left) or not reduced (right). Link 1 refers to the link protein of highest molecular weight.

Accumulation of [^3H]leucine-labelled macromolecules in the tissue

Cartilage sections were incubated with [^3H]leucine for up to 6 h and extracted with 4M-guanidinium chloride. The extracts were fractionated by direct dissociative CsCl-density-gradient centrifugation. By using a starting density of 1.36 g/ml, all proteins were recovered in the two top fractions (D3 + D4), whereas virtually all proteoglycans were present in the bottom D1 fraction (Heinegård *et al.*, 1981). An example of the fractionation obtained is shown in Fig. 2. The rates of [^3H]leucine incorporation into protein and proteoglycan were determined (Fig. 3). At all times more than 90% of the radioactivity was present in the protein fractions (D3 + D4), and about 5% was incorporated into proteoglycan core protein (D1). Labelling with a single radioactive amino acid does not necessarily give a correct measure of the relative rates of synthesis of different proteins, as their specific radioactivities may vary. Still, it appears certain that the proteoglycan core protein constitutes only a minor portion of the total protein synthesized.

Macromolecules in samples of the protein-rich D3 + D4 fractions were precipitated with ethanol and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels (Fig. 4). The 148 kDa protein, the link proteins and the 36 kDa protein were identified by comparison of electrophoretic mobilities of radioactive components with those of corresponding Kenacid Blue-stained bands (Fig. 4, lower frame), as well as with the mobilities of purified preparations of these proteins. To substantiate further the identification of the proteins, electrophoresis was done under reducing (Fig. 4, traces a–d) as well as under non-reducing (Fig. 4, traces e–h) conditions. Additional criteria for identity were obtained for the 148 kDa protein, where radiolabelled molecules co-migrated with the intact trimeric protein as well as with the reduced subunits. Both the 148 kDa protein and the 36 kDa protein could already be distinguished as major components after 1.5 h of pulse (Fig. 4, traces a and e). These proteins were best separated from other components by using non-reducing conditions for the electrophoresis. The major link protein could under reducing conditions be seen as a shoulder on the peak of the 148 kDa-protein subunits (Fig. 4, traces a–d), and more clearly as a separate peak under non-reducing conditions (Fig. 4, traces e–h). Some unidentified radiolabelled material with a mobility somewhat lower than that of the subunits of the 148 kDa protein was observed. It appears as two major peaks, more clearly visible after long labelling times (Fig. 4, traces c and d). The mobility of this material is not significantly changed by reduction. It seems to be of rather high specific radioactivity, as

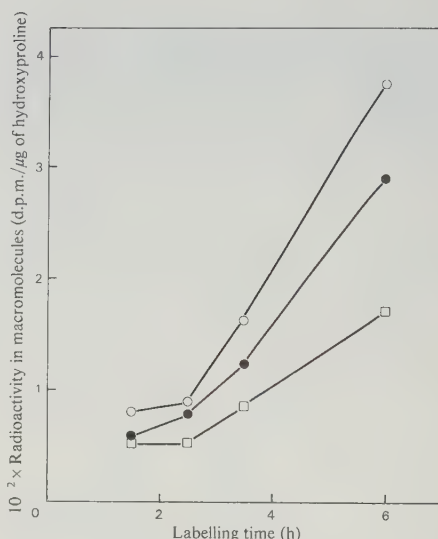


Fig. 5. Accumulation of individual [^3H]leucine-labelled proteins in the tissue

The rates of synthesis of the 148 kDa protein (●), the link proteins (□) and the 36 kDa protein (○) were calculated from sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms, indicated by the hatched areas in Fig. 4.

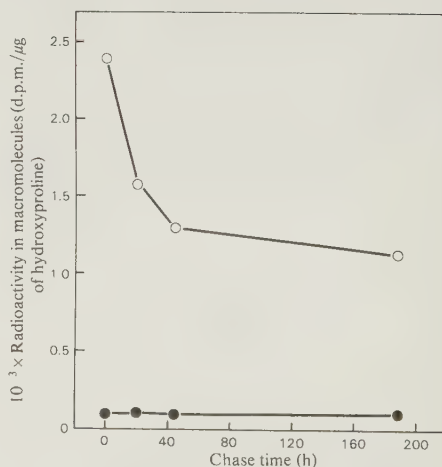


Fig. 6. Elimination of [^3H]leucine-labelled macromolecules from the tissue

The rate of elimination of proteoglycans (D1 fraction, ●) and proteins (pooled D3 and D4 fractions, ○) was determined as the decrease in [^3H]leucine during a non-radioactive chase of cartilage sections that had been prelabelled for 6 h.

this region of the gel is only weakly stained by Kenacid Blue (Fig. 4, lower frame).

The radioactivity corresponding to the 148 kDa protein, the link proteins and the 36 kDa protein was calculated as indicated in Fig. 4 (traces *e-h*) and plotted versus labelling time (Fig. 5). Even though the rather low amounts of radioactivity obtained with the shorter labelling times were difficult to determine with accuracy, the kinetics of the synthesis of the individual proteins appeared to be linear and were similar to those of the whole protein fraction and of the proteoglycan core protein. This indicates that neither of the proteins is a degradation fragment of other molecules. Most label was accumulated in the 36 kDa protein. It should, however, be noticed that this protein has a higher

content of leucine (about 140 residues/1000 residues) than any of the other proteins quantified (M. Paulsson, Y. Sommarin & D. Heinegård, unpublished work).

Elimination of [^3H]leucine-labelled macromolecules from the tissue

Cartilage sections were pulse-labelled with [^3H]leucine for 6 h and then 'chased' with non-radioactive Ham's F12 HA medium containing 2 mM-leucine for up to 188 h. The sections were extracted with 4 M-guanidinium chloride and centrifuged in CsCl density gradients as described above. A plot of [^3H]leucine label in guanidinium chloride-extractable proteins (D3 + D4) and proteoglycans (D1) as a function of chase time is shown in Fig. 6. No

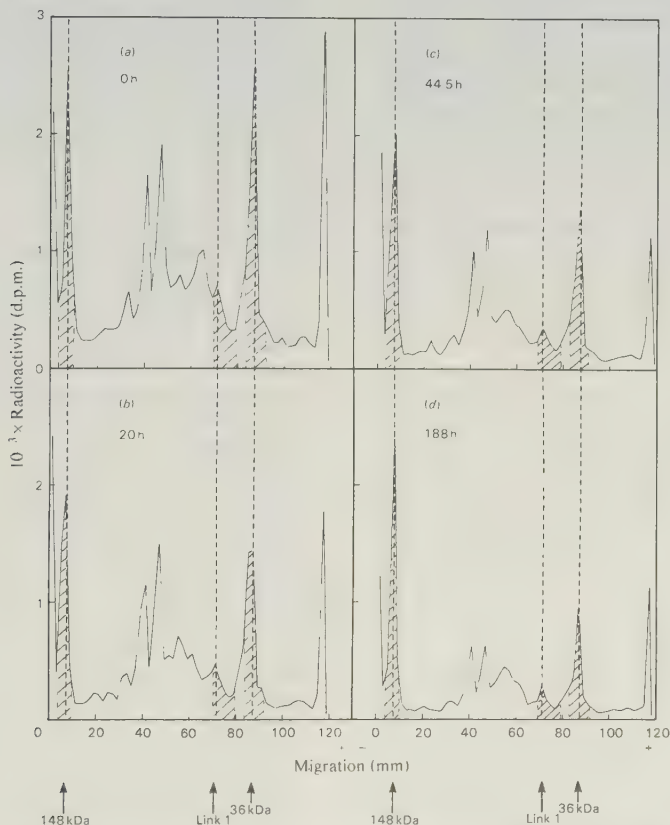


Fig. 7. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the extractable [^3H]leucine-labelled cartilage proteins after different periods of chase

The sample preparation and electrophoresis was as described in the legend to Fig. 4, but only radioactivity profiles of the non reduced samples are shown. The length of the non-radioactive chase is given for each sample in the Figure.

The hatched areas in the electrophoretograms were used for calculation of radioactivity in individual proteins.

significant turnover of proteoglycans was observed during an experimental period of about 1 week. In contrast, the radioactivity in proteins decreased to about half in this period. The decrease was especially marked during the first 50 h of the chase. Only small amounts of labelled macromolecules could be recovered from the medium. Therefore most of the decrease must be due either to degradation of labelled proteins to fragments too small to be precipitated with ethanol or possibly to integration of labelled proteins into the guanidinium chloride-insoluble material. The latter alternative is unlikely, as the radioactivity in the extraction residue decreased during the chase at a rate only slightly lower than that of the guanidinium chloride extract.

Samples of the protein-rich top fractions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The radioactivity profiles from the non-reducing gels (Fig. 7) were used to quantify individual proteins. The elimination rates of the various proteins were markedly different (Fig. 8). Whereas the 36 kDa protein had a relatively rapid turnover, the 148 kDa protein was eliminated very slowly, with a turnover rate more similar to that of

the proteoglycans. The slow elimination of the 148 kDa protein explains why larger amounts of this protein accumulate in tracheal cartilage (Fig. 4, lower frame), even though its rate of synthesis is similar to that of the other proteins. Interestingly, the link proteins, although quantified with greater error (cf. Fig. 7), appear to have a higher turnover rate than the proteoglycans. It is possible that excess link proteins synthesized are eliminated at a higher rate (Kimura *et al.*, 1980).

General discussion

Our initial experiments were aimed at identification of the 148 kDa and 36 kDa proteins in cultures of isolated bovine tracheal-cartilage chondrocytes, grown either as primary cells in suspension or in monolayer. Except for the use of chondrocytes from 6-month-old animals rather than foetuses, the culture conditions were those described previously (Björnsson & Heinegård, 1981). The cells were derived from a tissue known to contain large amounts of these proteins (Paulsson & Heinegård, 1982), and they retained the chondrocyte phenotype as expressed by production of cartilage proteoglycans and link proteins. Nevertheless we could demonstrate neither the 148 kDa protein nor the 36 kDa protein in the media or in guanidinium chloride extracts of the cells, by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In contrast, we could demonstrate their biosynthesis by using organ culture of cartilage sections, as described in the present paper. As the methodology used was identical except for the culture system, it appears that the synthesis of these proteins can be suppressed in isolated chondrocytes. Possibly production of the 148 kDa and 36 kDa proteins is induced by factors such as the interaction of the cells with a surrounding matrix.

Although having the advantage of allowing studies of cell function in an intact matrix, the use of cartilage sections presents some limitations. Thus in organ culture one problem is to minimize the variability between different cultures. Therefore several precautions were taken to achieve good repeatability in experiments. Sections were only taken from the central portion of tracheal rings from animals of a defined age. In most experiments measurements were based on pools of more than one section. Some variability in the mass of the cartilage sections can, however, not be eliminated. To correct for this variation, we found that hydroxyproline contents of the extraction residues provided reliable results, as could be expected since collagen is not likely to be lost to the medium, even during prolonged culture. By taking all these precautions, the relative rates of synthesis and turnover observed for the different macromolecules were highly reproducible between experiments.

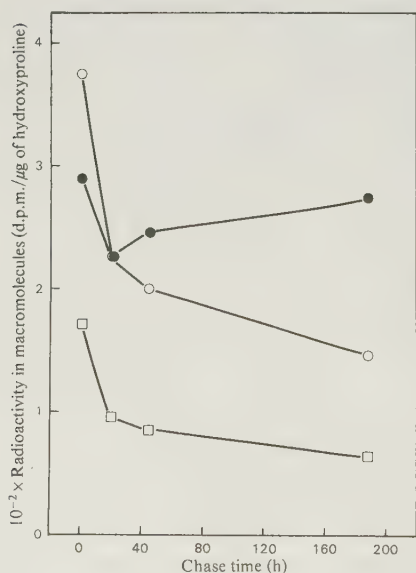


Fig. 8. Elimination of individual [^3H]leucine-labelled proteins from the tissue

The rates of elimination of the 148 kDa protein (●), the link proteins (□) and the 36 kDa protein (○) were calculated from sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms, as indicated by the hatched areas in Fig. 7.

One drawback remaining is that the extraction of labelled molecules is selective, i.e. some classes of proteins are extracted in good yield, whereas others are retained in the residue. It is therefore not possible to ascertain the nature of all the labelled material. Secondly, it is not possible to distinguish between matrix proteins and cellular proteins with the same certainty as when isolated cells are used. The kinetic data do, however, strongly indicate that most of the labelled proteins in the guanidinium chloride extracts are derived from the extracellular matrix. It should also be noted that the 148 kDa and 36 kDa proteins are major biosynthetic products of the tracheal-cartilage chondrocyte, each incorporating considerably more [^3H]leucine than does the cartilage proteoglycan core protein.

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A novel low-molecular-weight chondroitin sulphate proteoglycan isolated from cartilage

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Proteoglycans were isolated from cartilage by extraction with 4 M-guanidinium chloride followed by direct centrifugation in 4 M-guanidinium chloride/CsCl at a low starting density, 1.34 g/ml. *N*-Ethylmaleimide was included in the extraction solvent as a precaution against contamination of proteoglycans with unrelated proteins mediated by disulphide exchange. A novel, discrete, low-buoyant-density proteoglycan (1.40–1.35 g/ml) was demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Its proteoglycan nature was revealed by the shift in the molecular size observed on gel electrophoresis after treatment with chondroitinase ABC. The core protein was monodisperse. The proteoglycan was further purified by gel chromatography with and without addition of hyaluronate. The proteoglycan constitutes less than 2% (by weight) of the total extracted proteoglycans and is not capable of interacting with hyaluronate. The same proteoglycan was purified in larger quantities by sequential associative and dissociative CsCl-density-gradient centrifugation, zonal rate sedimentation in a sucrose gradient and gel chromatography on Sepharose CL-4B. The pure proteoglycan had a molecular weight of 76 300 determined by sedimentation-equilibrium centrifugation and an apparent partial specific volume of 0.59 ml/g. It contained about 25% protein (of dry weight) and had remarkably high contents of leucine and cysteine as compared with other proteoglycans. The proteoglycan contained two to three large chondroitin sulphate chains and some oligosaccharides.

Proteoglycans are major constituents of the cartilage intercellular matrix. A proteoglycan molecule has a central protein core to which polysaccharide side chains are attached via their reducing terminals. The cartilage proteoglycan, in particular, contains a very large number of chondroitin sulphate and keratan sulphate chains, attached to different regions of the protein core (Heinegård & Axelsson, 1977). Another region of the core protein, located at one end, has a structure allowing specific interaction with hyaluronic acid, the hyaluronic acid-binding region (Heinegård & Hascall, 1974; Heinegård & Axelsson, 1977). The core protein is very polydisperse, having a molecular weight of 200 000–300 000 (Hascall & Riolo, 1972; Hascall & Heinegård, 1974a). The intact proteoglycan monomers are also very polydisperse (Heinegård, 1977), with molecular weights ranging from less than one million to several millions, with an average of about 2.5×10^6 (Hascall & Sajdera, 1970).

The hyaluronic acid-binding region mediates a specific interaction between the proteoglycan and hyaluronic acid (Hardingham & Muir, 1973; Has-

call & Heinegård, 1974a,b; Hardingham *et al.*, 1976). Several proteoglycan monomers can bind to one molecule of hyaluronic acid, forming an extremely large proteoglycan aggregate. The aggregating proteoglycans, which contain the hyaluronic acid-binding region, constitute about 85% of the total extractable proteoglycans in cartilage. Another population, the non-aggregating proteoglycans, representing about 10%, cannot interact with hyaluronic acid (Heinegård & Hascall, 1979). These molecules do not contain the hyaluronic acid-binding region, although they contain more protein and less chondroitin sulphate (Heinegård & Hascall, 1979).

There have been reports of yet another population of proteoglycans of similar size to the non-aggregating proteoglycans, but containing little protein and mainly chondroitin sulphate (Mason & Mayes, 1973; Pearson & Mason, 1977). These proteoglycans, however, were isolated by techniques in which proteolysis was not minimized. A very slowly sedimenting proteoglycan of low buoyant density in CsCl density gradients has been

demonstrated as a sulphate-labelled component in extracts from embryonic cartilages and cell cultures (Kimata *et al.*, 1978). Swann *et al.* (1979) fractionated an extract of articular cartilage to sub-fractions containing molecules of very different sizes. One fraction contained small proteoglycans of a low buoyant density.

The present investigation was initiated in an attempt to design a procedure to isolate all extractable proteoglycans, under conditions preventing activity of degrading enzymes. It was considered important to isolate also proteoglycans of low buoyant density not bound into aggregates and therefore not recovered in the normal isolation procedure.

Materials and methods

Bovine nasal cartilage was obtained at the abattoir within a few minutes after slaughter. The cartilage was immediately carefully cleaned from surrounding tissue and perichondrium and sliced with a Surform blade. The cartilage slices were stored at -60°C until extraction. Cartilage slices were extracted for 24 h at 3°C with 12 vol. of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors 5 mM-benzamide hydrochloride, 0.1 M-6-amino-hexanoic acid and 10 mM-EDTA (Oegema *et al.*, 1975). In addition, the extraction solvent contained 4 mM-*N*-ethylmaleimide to prevent disulphide exchange and to inhibit any thiol proteinase. The extract was then filtered through Celite (BDH Chemicals, Poole, Dorset, U.K.).

CsCl-density-gradient centrifugation

The extract, containing about 6 mg of proteoglycan/ml, was adjusted to a density of 1.34 g/ml by addition of solid CsCl. It was then centrifuged in an MSE 8×25 ml angle rotor (22 ml per tube) at 35 000 rev./min (95 000 g, r_{av} , 6.886 cm) for 60 h at 18°C . By use of a tube piercer the tubes were emptied from the bottom to yield 11 fractions of volume 2 ml.

In other experiments identical extracts were dialysed against 10 vol. of 50 mM-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors listed above. After associative centrifugation (starting density 1.64 g/ml) as described elsewhere (Heinegård & Hascall, 1979), the top three-quarters of the tubes (A_{top} fraction) were recovered and dialysed against an equal volume of 8 M-guanidinium chloride. The density was adjusted to 1.35 g/ml by adding a small amount of solid CsCl. After centrifugation for 60 h as described above, the tubes were emptied in 2 ml fractions.

Densities of fractions were measured by using a

200 μl constriction pipette. Samples (25 μl) were removed for determination of uronic acid. Other samples were taken for electrophoresis on sodium dodecyl sulphate/polyacrylamide gels as described below. Care was taken so that each sample electrophoresed represented an equal portion of the original fraction. The fractions from the CsCl gradients were pooled and extensively dialysed against sodium acetate and distilled water and were finally freeze-dried.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Samples from the various fractionation procedures were dialysed into 20 mM-Tris/HCl buffer, pH 8.0. Equal portions were incubated with 0.002 unit of chondroitinase ABC (Miles Laboratories, Elkhart, IN, U.S.A.)/100 μl for 5 h at 37°C and without addition of enzyme. Before electrophoresis an equal volume of 2-fold-concentrated upper-reservoir buffer containing 10% (w/v) sucrose, 0.01% (w/v) EDTA and 10% (v/v) 2-mercapto-ethanol was added and the samples were incubated for 2 h at 37°C . In some cases the 2-mercapto-ethanol was omitted. Samples containing 10–50 μg of protein were electrophoresed on polyacrylamide gels in the buffer system of Neville (1971). The gels were T = 8.0% and C = 2.5%, where T is the percentage (w/v) of total monomer and C is the amount of *NN'*-methylenebisacrylamide expressed as fraction (% w/w) of total monomer (Neville, 1971). Gels were stained with Kenacid Blue R (BDH Chemicals).

In a separate experiment the purified proteoglycan was digested with chondroitinase ABC (0.004 unit/mg) and electrophoresed on polyacrylamide gels (T = 6–14%, C = 1%). Reference proteins (high-molecular-weight and low-molecular-weight standard proteins; Pharmacia Fine Chemicals, Uppsala, Sweden) were electrophoresed in an identical manner and the dependence of the relative mobility on the polyacrylamide concentration was determined, essentially as described by Banker & Cotman (1972).

Sucrose-gradient zonal rate sedimentation

The material that was recovered from the top fraction (A_{top}) of an associative CsCl density gradient (without guanidinium chloride), and then centrifuged in a dissociative CsCl density gradient (with guanidinium chloride) and recovered in the bottom fraction (A_{top} -D1 fraction), was extensively dialysed against water. Tris/HCl buffer (0.1 M), pH 7.5, was added to give a final concentration of 1 mM. The samples (2 ml) were layered on top of 14 ml of a 10–30% (w/v) linear sucrose gradient in 1 mM-Tris/HCl buffer, pH 7.5. After centrifugation at 50 000 rev./min (190 000 g, r_{av} , 6.886 cm) for 10 h

in an MSE 8 × 25 ml angle rotor the tubes were emptied and the absorbance of the effluent at 206 nm was recorded as described elsewhere (Franzén *et al.*, 1981).

Column chromatography

Columns (0.8 cm × 140 cm and 2.0 cm × 140 cm) of Sepharose CL-4B were eluted with 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8, and fractions of volume 1.5 and 10 ml respectively were collected. About 2.5 mg and 50 mg of sample respectively were applied to the columns. A Sepharose 4B column (1.6 cm × 140 cm) was eluted with 0.5 M-sodium acetate buffer, pH 5.8, and 4.9 ml fractions were collected. Approx. 15 mg of sample was applied. Samples to be chromatographed were dissolved in 4 M-guanidinium chloride and dialysed against the solvent used for chromatography. A column (0.6 cm × 180 cm) of Bio-Gel P-30 was eluted with 0.25 M-pyridinium acetate buffer, pH 6.5. Fractions of volume 0.8 ml were collected.

Enzymic treatment

To test for the presence of dermatan sulphate and chondroitin sulphate, equal samples of the purified proteoglycan were dissolved in 0.1 M-Tris/HCl buffer, pH 8.0, and digested with 0.05 unit of chondroitinase AC-II and chondroitinase ABC/mg respectively for 6 h at 37°C. The samples were separately chromatographed on Bio-Gel P-30.

Samples were digested for 5 h at 60°C with papain (2 × crystallized and containing about 25 mg/ml of protein; Sigma Chemical Co., St Louis, MO, U.S.A.) (1 µl/mg of proteoglycan) in 50 mM-sodium phosphate buffer, pH 6.5, containing 10 mM-EDTA and 5 mM-cysteine hydrochloride. The digests were then either chromatographed on Sephadex G-200 to reveal the size of the side chains or chromatographed on ECTEOLA-cellulose to isolate the glycosaminoglycans (Axelsson & Heinegård, 1975).

Analytical ultracentrifugation

Molecular weights were determined by sedimentation-equilibrium centrifugation by the high-speed method of Yphantis (1964). The sample was dissolved in and extensively dialysed against 4.0 M-guanidinium chloride (Ultrapure; Schwarz-Mann, Orangeburg, NY, U.S.A.)/5 mM-Tris/HCl buffer, pH 7.0. An MSE Centriscan analytical ultracentrifuge was used and the distribution of the material at equilibrium was determined both by using the schlieren system and by using the photoelectric scanner and a 280 nm interference filter. Apparent partial specific volume of the proteoglycan in 4 M-guanidinium chloride was determined by using the $^2\text{H}_2\text{O}$ method (Thomas & Edelstein, 1971). The apparent molecular weight of the proteoglycan in guanidinium chloride/ $^2\text{H}_2\text{O}$ was corrected by the

factors 1.0155 for the protein and 1.0158 for the glycosaminoglycan part to compensate for exchangeable hydrogen atoms being replaced by deuterium.

Sedimentation coefficients were determined by using the MSE Centriscan analytical ultracentrifuge. Samples were centrifuged in buffered 4.0 M-guanidinium chloride (as above) at 59 000 rev./min. Scans at 280 nm were taken at regular intervals by using the photoelectric scanner. Sedimentation coefficients were calculated in accordance with the MSE manual (Technical Publication, Supplement no. 73), corrected to 20°C and water and extrapolated to zero concentration.

Chemical methods

Uronic acid contents of fractions from the density gradients and column effluents were determined by the carbazole method (Bitter & Muir, 1962). When columns were eluted with 0.5 M-sodium acetate buffer or 0.25 M-pyridinium acetate buffer, the uronic acid content was determined by an automated carbazole procedure (Heinegård, 1973).

Sialic acid content was determined by the method of Jourdain *et al.* (1971).

Hexosamine contents were determined after hydrolysis of samples in 4 M-HCl at 100°C for 10 h under argon. Amino acid contents were determined after hydrolysis of samples in 6 M-HCl at 110°C for 24 h under argon. The HCl used was of AristaR quality (BDH Chemicals). A Durrum automatic amino acid analyser was used for both hexosamine and amino acid determinations.

Neutral sugar content was determined by ion-exchange chromatography of borate complexes of monosaccharides as described by Walborg & Kondo (1970) and modified by S. Lohmander (personal communication). The sample was hydrolysed in 2 M-trifluoroacetic acid for 2 h at 100°C.

Results and discussion

Isolation of proteoglycans under denaturing conditions

One of the objects of the present work was to develop a method for isolation of all proteoglycans in cartilage under conditions minimizing degradation. Therefore in initial experiments the extract was centrifuged directly in 4 M-guanidinium chloride, avoiding the non-denaturing conditions of re-association. One problem encountered was that, with the commonly used starting density of 1.5 g/ml, some proteoglycans will under dissociative conditions be recovered in or near the top fraction of the gradient, as is discussed elsewhere (Heinegård, 1977). Therefore we decided to use the much lower starting density of 1.34 g/ml. The density obtained in the bottom fraction, then, is actually about the same

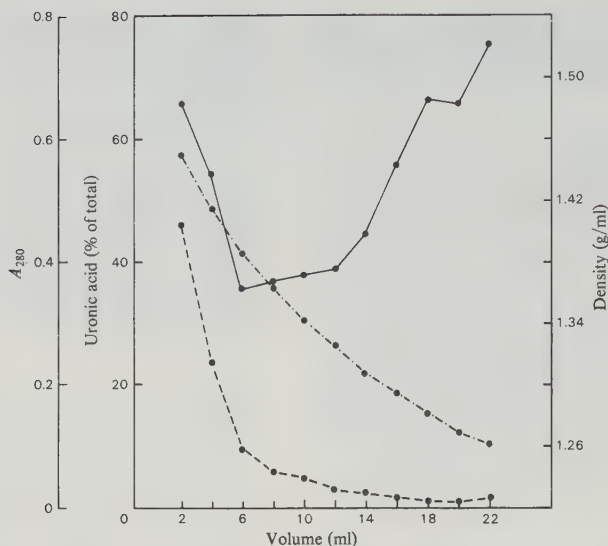


Fig. 1. Guanidinium chloride (4M)/CsCl-density-gradient centrifugation (starting density 1.34 g/ml) of cartilage extract. Experimental details are given in the text. —, A_{280} ; ----, A_{530} (carbazole reaction) as percentage of total in the gradient; - · - · -, density.

as that obtained for the top fraction of a gradient with a starting density of 1.52 g/ml. The bottom third of the centrifuge tube contained more than 85% of the uronic acid in the gradient (Fig. 1) and about 80% of the recovered material (by weight).

In order to determine possible contamination with non-proteoglycan proteins, equal portions of the fractions (pooled two and two from the bottom) were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with and without reduction (Figs. 2a and 2b). No Kenacid-staining protein bands were observed in the bottom two pools analysed, i.e. the bottom 8 ml. It appears, then, that proteoglycans can be purified in good yield by direct CsCl-density-gradient centrifugation of 4M-guanidinium chloride extracts at low starting density. In preliminary experiments, when extraction was performed, without *N*-ethylmaleimide in the extraction solvent, protein bands were occasionally observed in reduced samples of the bottom fraction. It is possible that proteoglycans may form complexes with other proteins through disulphide exchange, which may be favoured by the denaturing conditions used for extraction. Proteins bound to the proteoglycans will sediment to the bottom of the gradients. Because of such results it was considered important to include *N*-ethylmaleimide to block free thiol groups in all extraction solvents used.

Equal portions of the fractions from the CsCl-density gradient centrifugation were digested with chondroitinase ABC to remove chondroitin sulphate side chains. They were then electrophoresed with and without reduction (Figs. 2c and 2d). The amount of enzyme used did not give any detectable bands. In separate experiments it was shown that the chondroitinase used did not contain detectable proteolytic activity, when [3 H]acetylated haemoglobin was used as a substrate. The fraction (D2) obtained from a density of 1.40–1.35 g/ml contained a protein band with a mobility close to that of the link proteins (Keiser *et al.*, 1972; Hascall & Heinegård, 1974a). Since the chondroitinase treatment had only removed the chondroitin sulphate side chains, it was considered probable that this band represents the core protein of a low-buoyant-density small proteoglycan. It is noteworthy that the protein core is monodisperse, in contrast with the extreme polydispersity of the high-buoyant-density proteoglycans. In a separate experiment samples of all the fractions of an identical density gradient were digested with chondroitinase ABC and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. The gel patterns showed the core protein as a band with a mobility intermediate between those of the two major link proteins (results not shown).

All the other proteins extracted from the cartilage

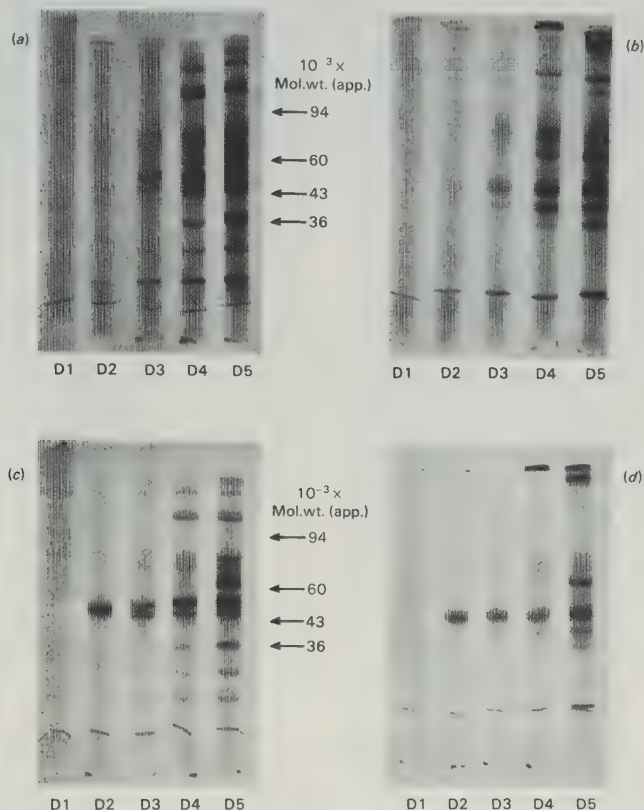


Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of fractions (4 ml) from 4 M-guanidinium chloride/CsCl-density-gradient centrifugation

Experimental details are given in the text. (a) Reduced samples; (b) non-reduced samples; (c) samples digested with chondroitinase, reduced; (d) samples digested with chondroitinase, non-reduced.

were also present in the gels. The fact that the link proteins were found as far from the top fractions as in fraction D3 is important. This may be a result of the re-orientation of the gradient in the angle rotor at the end of centrifugation. The matrix protein previously isolated from tracheal cartilage (Paulsson & Heinegård, 1979, 1981) was, however, present only in the top fraction of this nasal-cartilage preparation (Fig. 2). It is possible that the link protein is not at equilibrium at the top of the gradient.

The two fractions that contained the major proportion of the low-buoyant-density proteoglycan (corresponding to D2 fraction in Fig. 2) were pooled, extensively dialysed against sodium acetate buffer and distilled water and then freeze-dried. The

material recovered constituted about 6% of the total in the gradient (by weight). The bottom fractions, which contained only high-buoyant-density large proteoglycans, weighed 12.5 times as much as the low-buoyant-density small proteoglycan fraction.

A sample (50 mg; d-D2 fraction) was chromatographed on the preparative Sepharose CL-4B column eluted with 4 M-guanidinium chloride (Fig. 3). Two major peaks were observed. A uronic acid-rich peak was eluted at the void volume, and a protein-rich peak was eluted well included. The effluent fractions were pooled as indicated in Fig. 3. A total of 48 mg was recovered, of which 26 mg was from the protein-rich peak. Samples, representing equal portions of the fractions, were taken for chondroitinase ABC digestion and sodium dodecyl

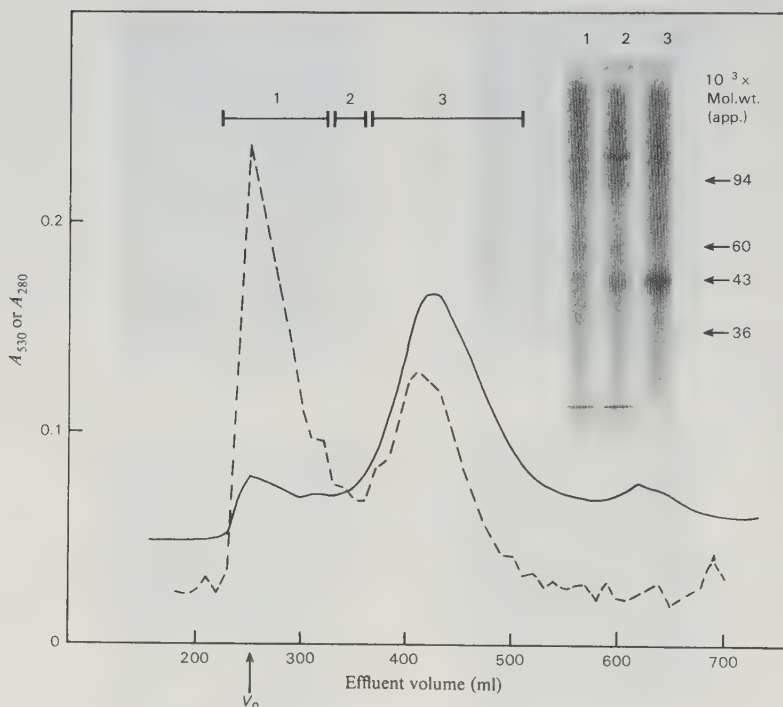


Fig. 3. Sepharose CL-4B-chromatography (eluent 4 M-guanidinium chloride) of the bottom fraction from 4 M-guanidinium chloride/CsCl-density-gradient centrifugation

Experimental details are given in the text. —, A_{280} ; ----, A_{530} (carbazole reaction). The insert shows the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis patterns of samples (80 μ g) of the indicated fractions after chondroitinase digestion. V_0 , Void volume.

sulphate/polyacrylamide-gel electrophoresis after reduction (Fig. 3). The band corresponding to the low-buoyant-density proteoglycan was observed only in the gel corresponding to the protein-rich included peak (d-D2-4B2 fraction). The material in the void volume, then, probably represents low-buoyant-density proteoglycans of the polydisperse population representing the bulk of the proteoglycans in cartilage.

Preliminary experiments showed that the proteoglycan preparation contained some material that reacted with antibodies directed against the hyaluronic acid-binding region of regular cartilage proteoglycan monomers (results not shown). Molecules containing the hyaluronic acid-binding region are capable of interacting with hyaluronic acid. Therefore it should be possible to shift the elution position of such molecules to the void volume by the addition of very-high-molecular-weight hyaluronic acid, which in itself chromatographs excluded from the gel. A sample (15 mg) of the low-buoyant-density

small proteoglycan, purified by Sepharose CL-4B chromatography (d-D2-4B2 fraction), was dissolved in 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. After dialysis against 500 vol. of 0.5 M-sodium acetate buffer, pH 5.8, high-molecular-weight hyaluronic acid (Healon, a gift from Pharmacia Fine Chemicals) was added (1%, by weight) and the sample was incubated overnight at 4°C. It was then chromatographed on a column of Sepharose 4B eluted with 0.5 M-sodium acetate buffer, pH 5.8 (Fig. 4). A relatively more-protein-rich peak was eluted in the void volume of the column, and a more-uronic acid-rich component was eluted well included. Fractions were pooled as indicated in Fig. 4, dialysed against sodium acetate buffer and distilled water, and freeze-dried. The included component constituted 52% (w/w) of the material recovered. Equal portions of each fraction were digested with chondroitinase ABC and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels. A strongly staining band corresponding

to the low-buoyant-density small proteoglycan was present in the gel containing the sample from the uronic acid-rich included peak. This gel also showed a minor contaminating protein component with a lower mobility (Fig. 4). The molecular weight of the material in the purified proteoglycan fraction was determined by sedimentation-equilibrium centrifugation. After extrapolation to zero concentration, values of 67600 and 70200 were obtained by scanning at 280nm and with the schlieren optics

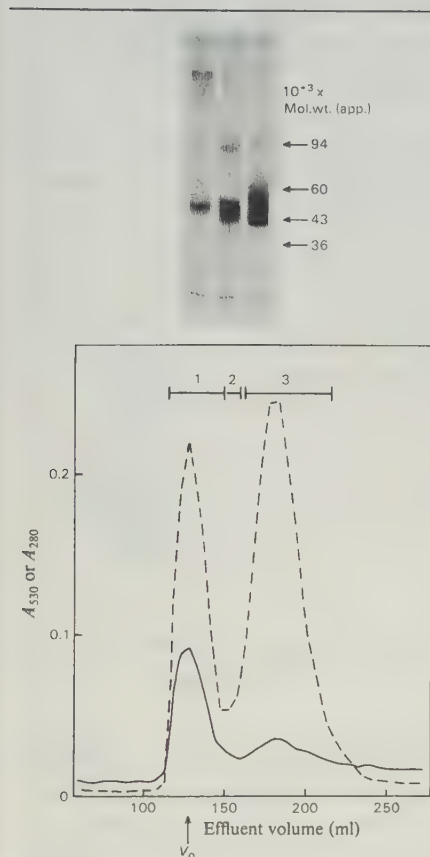


Fig. 4. Sepharose 4B chromatography (eluent 0.5M-sodium acetate buffer, pH5.8) of the included fraction from dissociative chromatography of the bottom fraction from 4M-guanidinium chloride/CsCl-density-gradient centrifugation (d-D1-4B2 fraction)

Experimental details are given in the text. —, A_{280} ; ----, A_{530} (carbazole reaction). At the top is shown the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis patterns of samples (100 μ g) of the indicated pools with and without chondroitinase digestion. V_0 , Void volume.

respectively. A value for apparent partial specific volume of 0.59 ml/g was used, as discussed below. The lower value obtained with the scanning at 280nm may reflect the presence of a low-molecular-weight protein contaminant. In support, the plots of $\ln c$ versus r^2 were non-linear at the lower concentration range.

Isolation of the small proteoglycan after associative CsCl-density-gradient centrifugation

To purify the proteoglycan further and in larger amounts, an alternative procedure was devised. Cartilage was extracted with 4M-guanidinium chloride, and proteoglycans in the extract were reassociated essentially as described elsewhere (Heinegård & Hascall, 1979), the only difference being that *N*-ethylmaleimide was included in the solvent to prevent disulphide exchange. After associative CsCl-density-gradient centrifugation, the top three-quarters of the tube (A_{top} fraction) contained all of the small proteoglycan and a proportion of other types of cartilage proteoglycans. To separate proteoglycans from proteins, this material was centrifuged under the dissociative conditions of 4M-guanidinium chloride in a CsCl gradient with the starting density of 1.35 g/ml. Equal portions of the fractions were then dialysed into water and freeze-dried. Each portion was digested with chondroitinase ABC, reduced and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels (Fig. 5). The bottom pool (6 ml; A_{top} -D1 fraction), which contained the major portion of the small proteoglycan, as indicated by the presence of the core protein, was dialysed into water. This material was about 10% (by weight) of the A1

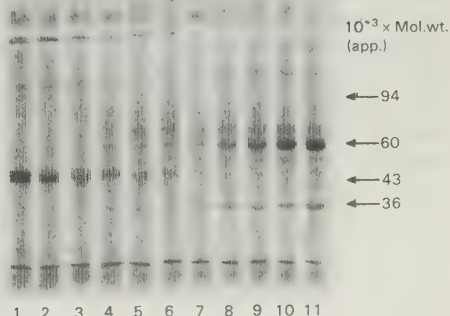


Fig. 5. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of chondroitinase-digested and reduced fractions from sequential associative-dissociative CsCl-density-gradient centrifugation of cartilage extracts

For experimental details see the text.

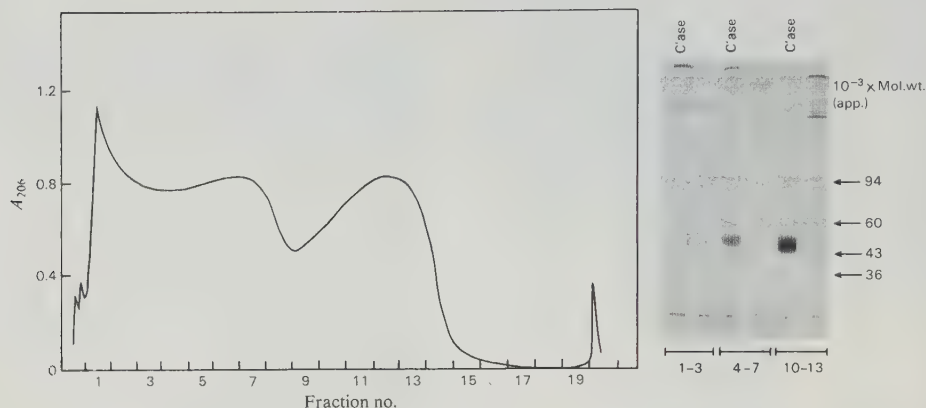


Fig. 6. Zonal rate centrifugation in 10–30% (w/v) sucrose in 1 mM-Tris/HCl buffer, pH 7.5, of the bottom fraction from sequential associative–dissociative CsCl-density-gradient centrifugation (A_{top} -D1 fraction)

Experimental details are given in the text. Samples (50 μ g) of indicated fractions were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels before and after chondroitinase (C'ase) digestion. —, A_{206} .

fraction from the same preparation. Samples were subjected to zonal rate centrifugation in sucrose density gradients, to separate the small proteoglycans from any larger cartilage-type proteoglycans present. In preliminary experiments it was shown that optimal separation was obtained when the salt concentration in the gradient was kept as low as possible. The tracing of the absorbance at 206 nm of the tube contents showed at least two major components, as well as the presence of material that had sedimented to the bottom (Fig. 6). Samples of the indicated fractions were recovered and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis before and after chondroitinase-ABC digestion (Fig. 6). The major portion of the small proteoglycan was present in the slowest-sedimenting component (A_{top} -D1-SS2 fraction). The material in this fraction was recovered after dialysis against water and freeze-drying. It contained about 25% (by weight) of all material recovered from the zonal rate centrifugation. This preparation was then dissolved in 4 M-guanidinium chloride and chromatographed on a Sepharose CL-4B column eluted with the same solvent. One component was eluted in the void volume (Fig. 7), and another component was eluted included. The indicated fractions were recovered. The included material (A_{top} -D1-SS2-4B2 fraction) represented about 55% (by weight) of the material applied to the column. Samples of the two pools were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with and without prior digestion with chondroitinase ABC (Fig. 7). It appears that the included fraction contained only the small proteoglycan. No bands other than

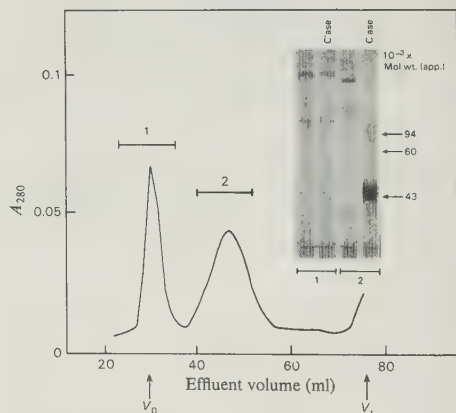


Fig. 7. Sepharose CL-4B chromatography (eluent 4 M-guanidinium chloride) of the fraction containing the small proteoglycan recovered from zonal rate centrifugation in a sucrose density gradient (cf. Fig. 6)

Experimental details are given in the text. Samples (50 μ g) of indicated fractions were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels before and after chondroitinase (C'ase) digestion. —, A_{280} . V_0 , Void volume; V_t , total volume.

the core protein were observed in the two gels of the included proteoglycan component, indicating purity of the proteoglycan. The excluded fraction contained a minor component with a much lower mobility, which was not affected by chondroitinase

digestion, indicating that it represents a molecule not containing galactosaminoglycans. This component had the same mobility as the impurity of the dissociatively prepared small proteoglycan discussed above. In addition, both the gels contained material not entering the gel, probably high-molecular-weight proteoglycans.

Analysis of the small proteoglycan

The preparation of the small proteoglycan was considered pure and was further analysed. Its molecular weight was determined to be 76 300 by sedimentation-equilibrium centrifugation in 4 M-guanidinium chloride (Fig. 8). Identical results were obtained by using the schlieren optics and by scanning at 280 nm. This value is in agreement with those obtained for the dissociatively prepared proteoglycan (see above). Consequently the molecule can be prepared by a scheme including non-denaturing conditions as well as when maintaining denaturing conditions during the preparation to abolish degradation. The molecular weight represents a lowest value, since molecular weights of polydisperse molecules are usually underestimated when the meniscus-depletion method is used. It should be stressed, however, that the same values were obtained when centrifugation was done at 15 000–20 000 rev./min, indicating that the effects of the

polydispersity were minor. The apparent partial specific volume in 4 M-guanidinium chloride was determined to be 0.59 ml/g. Owing to the complexity of assessing the partial specific volume of proteoglycans, this value should be considered an estimate. Sedimentation-velocity centrifugation in 4 M-guanidinium chloride yielded an $s_{20,w}^0$ value of 3.11 S.

The proteoglycan was further characterized (Table 1). Approx. 25% of the dry weight was protein. The amino acid composition was quite different from that of other cartilage proteoglycans (Heinegård & Hascall, 1979). The contents of serine and threonine were low, indirectly indicating a relatively low glycosaminoglycan content. In contrast, the content of leucine was very high and that of cysteine was comparatively high. The amino acid composition is similar to that previously described for a keratan sulphate proteoglycan with a similar molecular weight, which was isolated from bovine cornea (Axelsson & Heinegård, 1978). The proteoglycan had lower contents of hexosamines than did

Table 1. Amino acid and carbohydrate composition of the low-molecular-weight cartilage proteoglycan
For experimental details see the text.

	Composition	
	(nmol/mg)	(residues/ 1000 residues)
Aspartic acid	309	129
Threonine	94	39
Serine	158	66
Glutamic acid	234	98
Proline	173	72
Glycine	170	71
Alanine	121	50
Cysteine	45	19
Valine	128	53
Methionine	2	1
Isoleucine	131	55
Leucine	342	143
Tyrosine	66	28
Phenylalanine	81	34
Histidine	86	36
Lysine	154	64
Arginine	107	44
Glucosamine	65	
Galactosamine	841	
Uronic acid	917	
Mannose	33	
Galactose	27	
Fucose	14	
Xylose	42	
Glucose	17	
Sialic acid	Not detected	
Protein (% dry wt.)		25.2
Hexosamine (% dry wt.)		16.2
Uronic acid (% dry wt.)		17.8

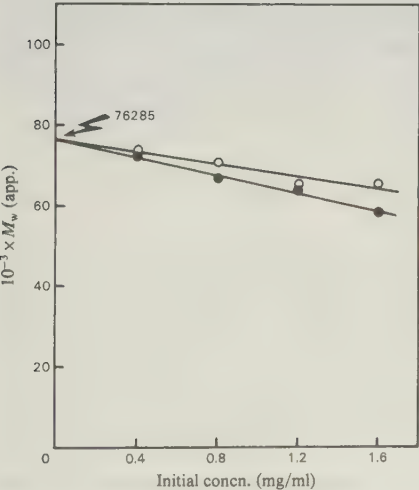


Fig. 8. Determination of the molecular weight of the small proteoglycan by sedimentation-equilibrium centrifugation
Experimental details are given in the text. Extrapolation was to zero concentration. O, Schlieren; ●, scanning at 280 nm.

other cartilage proteoglycans. Galactosamine was the major hexosamine. In separate experiments the glycosaminoglycans were isolated by ion-exchange chromatography of a papain digest of the proteoglycan (Axelsson & Heinegård, 1975). They contained only galactosamine, indicating that the proteoglycan contained only galactosaminoglycans and that the glucosamine was present in oligosaccharides attached to the protein core. In support, the proteoglycan contained mannose and fucose, not present in the glycosaminoglycan side chains. The low content of galactose may be due to losses during hydrolysis. It is notable, however, that the molar ratio of xylose to galactosamine is much lower than would be expected from the large chondroitin sulphate side chains (discussed below). It is possible that the proteoglycan contains some xylose residues not further substituted with a glycosaminoglycan side chain. The glucose present may be due to some sucrose remaining from the zonal rate centrifugation.

The character of the galactosaminoglycan side chains was determined. One sample of the proteo-

glycan was digested with chondroitinase AC-II and another identical sample was digested with chondroitinase ABC. The two chromatograms were identical (Fig. 9), and virtually all of the uronic acid was eluted in the total volume of the column. Consequently, the side chains were chondroitin sulphate that could be completely depolymerized by the action of chondroitinase AC.

The chondroitin sulphate was liberated by papain digestion of the proteoglycan and chromatographed on Sephadex G-200 (Fig. 10). The chains were eluted less retarded than a control sample of chondroitin sulphate isolated from a preparation of bovine nasal-cartilage proteoglycan monomer (A1-D1 fraction).

The protein content of the small proteoglycan indicated that the molecular weight of the protein core should be about 20000. This value is considerably lower than the apparent molecular weight observed on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the core preparation after chondroitinase-ABC digestion. Therefore the dependence of the electrophoretic mobility of the core protein on the concentration of polyacrylamide was determined over the range 6–14%. By using the theoretical approach of Ferguson (1964), retardation coefficients (K_R) and apparent free electrophoretic mobilities (M_0) could be calculated for the core protein and for a number of reference proteins. A plot of K_R versus molecular weight of reference proteins was linear, and from this relationship an apparent molecular weight of the core protein of the small proteoglycan of 42300 was obtained. Banker & Cotman (1972) have suggested plots of M_0 versus K_R as a sensitive test of ideality of the behaviour of proteins in sodium dodecyl sulphate/polyacryl-

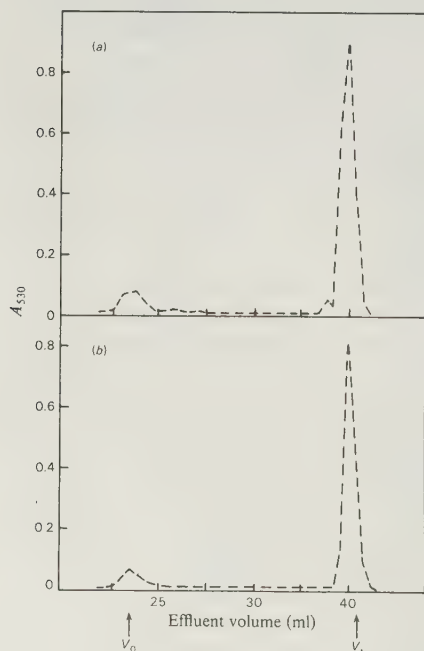


Fig. 9. Bio-Gel P-30 chromatography of chondroitinase AC-II (a) and chondroitinase ABC (b) digests of the small proteoglycan

Experimental details are given in the text. ———, A_{530} (carbazole reaction).

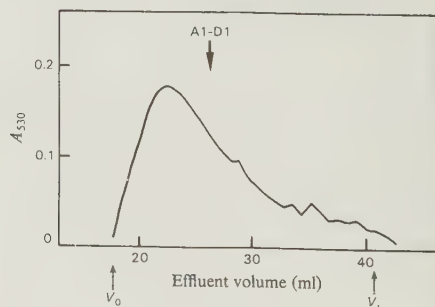


Fig. 10. Sephadex G-200 chromatography of a papain digest of the small proteoglycan to determine size distribution of side chains

Experimental details are given in the text. The elution position of chondroitin sulphate side chains of large cartilage proteoglycans (A1-D1 fraction) is indicated. ———, A_{530} (carbazole reaction).

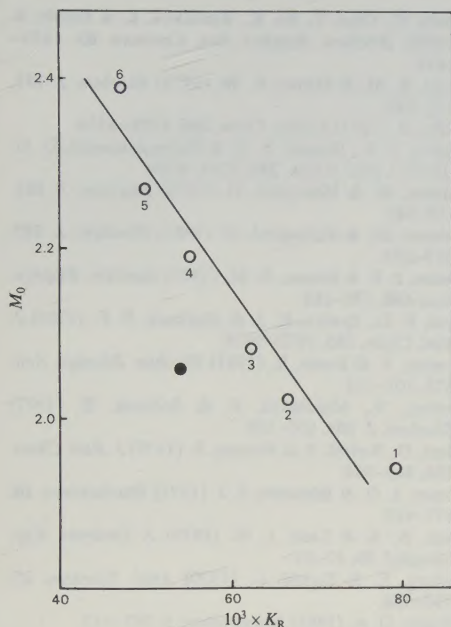


Fig. 11. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis at various concentrations of polyacrylamide of chondroitinase-digested and reduced small proteoglycan

Experimental details are given in the text. Plots of the free electrophoretic mobility versus retardation coefficient of reference proteins (○) and of the core protein of the small proteoglycan (●) are shown. Reference proteins were: phosphorylase *b* (1), bovine serum albumin (2), catalase subunit (3), ovalbumin (4), lactate dehydrogenase subunit (5) and carbonic anhydrase (6).

amide-gel electrophoresis. Such a plot indicated that the core protein had a lower apparent free electrophoretic mobility than should be expected from its retardation coefficient, when compared with the reference proteins (Fig. 11). Consequently, its behaviour on electrophoresis is non-ideal and would result in too high estimates of the molecular weight. The aberration could at least partly be due to the presence of oligosaccharides.

General discussion

The novel proteoglycan isolated is much smaller than and qualitatively different from previously described proteoglycans. It probably occurs in most types of cartilage. By using sodium dodecyl sul-

phate/polyacrylamide-gel electrophoresis with and without previous digestion with chondroitinase ABC it has also been identified in bovine tracheal cartilage and in different layers of bovine hip articular cartilage (results not shown). Since the latter cartilage does not contain blood vessels in its superficial layers, the small proteoglycan discussed cannot be derived from blood vessels. Furthermore, Kasarawa *et al.* (1979) and Vasan & Lash (1979) observed a slowly sedimenting proteoglycan in chondrocyte and tissue-culture experiments, most probably corresponding to the small proteoglycan. Swann *et al.* (1979) prepared a fraction from articular cartilage containing a major proportion of a small proteoglycan. This material had a molecular weight of 240 000 and high contents of leucine, and probably represents a fraction enriched in the small proteoglycan discussed. Stanescu *et al.* (1977) identified and later Stanescu & Sweet (1981) isolated from baboon cartilage a low-buoyant-density proteoglycan with a high relative mobility on electrophoresis on composite agarose/polyacrylamide gels. This material had an amino acid composition similar to that of the small proteoglycan discussed in the present paper.

The function of the small proteoglycan is unclear. Undifferentiated mesenchyme, however, appears to contain a higher proportion of molecules of this kind (Kimata *et al.*, 1978; Vasan & Lash, 1979; Royal *et al.*, 1980). The amino acid composition is quite different from that of the large cartilage proteoglycans. The chondroitin sulphate chains are considerably larger. Therefore it is unlikely that the small proteoglycan represents a catabolic product of the large proteoglycans. The fact that it can be isolated under denaturing conditions indicated that it is not a product of degradation during isolation. It is probable that the small proteoglycan contains two chondroitin sulphate chains and a few oligosaccharides.

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